Stimulation of Biosynthesis of Tobacco Mosaic Virus by Antimetabolites

By EVAMARIE SANDER

Max-Planck-Institut für Virusforschung, Biochemische Abteilung,
Tübingen, Germany

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SUMMARY

Tobacco leaf discs were treated with different concentrations of actinomycin C, D,L-parafluorphenylalanine, 8-azaguanine, chloramphenicol and puromycin-dihydrochloride before, simultaneously and after inoculation with tobacco mosaic virus. The treatment with low concentrations of antimetabolite simultaneously with tobacco mosaic virus inoculation stimulated the tobacco mosaic virus biosynthesis up to ninefold, whereas higher concentrations caused a ten- to 1000-fold inhibition. The stimulatory effect of pretreatment suggests the presence of a factor or factors with antiviral properties present in plant tissue becoming activated upon virus infection. The lack of an effect of treatment with antimetabolites 3 to 30 hr after inoculation indicates that activation of such factor(s) must occur within the first 30 min. after infection of tobacco leaf cells with tobacco mosaic virus.

INTRODUCTION

The experimental evidence for the sequence of steps leading to the synthesis of tobacco mosaic virus (TMV) in the tobacco leaf is still rather scanty. From the experiments of Engler & Schramm (1958) it is known that the removal of the protein coat is one of the first steps in TMV infection. With antimetabolites inhibiting nucleic acid or protein metabolism it should be possible to block certain steps in the process of infection. Therefore, actinomycin, D,L-parafluorphenylalanine, 8-azaguanine, chloramphenicol and puromycin were applied to tobacco leaf discs simultaneously with TMV and before and after inoculation. The rate of virus synthesis was measured by two different bioassays. Besides the expected inhibition at high concentration a stimulation of biosynthesis was observed at low concentrations of the antimetabolites indicating a yet unidentified function of the virus in its biosynthesis.

METHODS

Virus. TMV strain VULGARE (Max-Planck-Institut für Virusforschung, Tübingen) was always used in a concentration of $5 \times 10^{-6}$ g./ml. aqueous solution. The virus concentration was calculated from the content of N$_{6}$/ml. virus solution (Markham, 1959). Throughout the experiments, the virus was applied to leaves with a frosted-glass spatula in the presence of carborundum, 200 mesh.

Hosts. Nicotiana tabacum L. var. Xanthi necroticum served as local lesion host and var. Samsun as systemic host.
Antimetabolites. Samples of actinomycin C and chloramphenicol sodium monosuccinate (Leukomycin) were received as gifts from Farbenfabriken Bayer AG, Leverkusen, Germany. Puromycin-dihydrochloride and DL-parafluorphenylalanine were obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A., and 8-azaguanine from the California Foundation for Biochemical Research, Los Angeles, California, U.S.A.

Nutrient solution. For the application to leaf discs the antimetabolites were dissolved in a Vickery solution diluted 1/2 with 0.025 M-phosphate buffer pH 6.4 throughout the experiments. The concentrated solution contained in 1 l. of 0.025 M-phosphate buffer, 0.001 M-CaCl₂, 0.002 M-MgSO₄, and 0.002 M-(NH₄)₂SO₄. The pH was adjusted to 6.4 (Vickery et al. 1937).

Test on Samsun and Xanthi tobacco plants to measure TMV-synthesis in leaf discs treated with antimetabolites immediately following virus inoculation. Leaves of Samsun tobacco plants in the four-leaf stage were inoculated with TMV. The leaves were then rinsed with water, ten leaves stacked and discs of 1.2 cm. cut out. Twenty discs were randomly selected and placed, leaf-surface up, into glass Petri dishes of 8.5 cm. diameter. The dishes contained 10 ml. Vickery solution with varying concentrations of the different antimetabolites (Table I). The steps from inoculation of leaf discs to exposure to antimetabolites were executed within 3 min. The Petri dishes were then placed in a temperature- and light-controlled chamber (23 ± 1°, 3500 lux). As controls, leaf discs were kept in Vickery solution without the antimetabolites.

After incubation times of 0, 30, 48, 72 and 96 hr two Petri dishes with antimetabolites

Table 1. Dilution factors for homogenates of tobacco leaf discs inoculated with tobacco mosaic virus and treated with antimetabolites

<table>
<thead>
<tr>
<th>Treatment (hr)</th>
<th>Chloramphenicol (µg./ml.)</th>
<th>Puromycin (µg./ml.)</th>
<th>Controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1/5</td>
<td>1/5</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>1/5</td>
<td>1/5</td>
<td>10</td>
</tr>
<tr>
<td>48</td>
<td>1/5</td>
<td>1/5</td>
<td>50</td>
</tr>
<tr>
<td>72</td>
<td>1/100</td>
<td>1/100</td>
<td>500</td>
</tr>
<tr>
<td>96</td>
<td>1/200</td>
<td>1/200</td>
<td>1,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment (hr)</th>
<th>Actinomycin (µg./ml.)</th>
<th>p-Fluorophenylalanine (µg./ml.)</th>
<th>8-Azaguanine (µg./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>30</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>48</td>
<td>1/5</td>
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</tr>
<tr>
<td>72</td>
<td>1/100</td>
<td>1/100</td>
<td>1/100</td>
</tr>
<tr>
<td>96</td>
<td>1/100</td>
<td>1/100</td>
<td>1/100</td>
</tr>
</tbody>
</table>

* Leaf discs in nutrient solution without antimetabolite treatment, but inoculated with TMV.
† All antimetabolites were dissolved in Vickery solution diluted 1/2 with 0.025 M-phosphate buffer pH 6.4.
‡ All dilutions were made with 0.025 M-phosphate buffer pH 7.2.
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as well as two control dishes were removed from the incubation chamber and the leaf discs alternately blotted and rinsed in water 3 times. The blotted discs were stored at -20° until all samples could be assayed on the same batch of Xanthi tobacco plants. For this test each sample was homogenized in the presence of 4 ml. 0.025 M-phosphate buffer pH 7.2 with a pestle in a mortar in an icebath. From the 20 discs/plate approximately 1 ml. of sap was obtained which was thus diluted 1/5. Depending on incubation time and antimetabolite concentration the dilution of plant sap fell within the range of 1/5 to 1/200 (for dilution scheme see Table 1). From the diluted sample 0.3 ml. was applied to 4 Xanthi tobacco plants with 4 leaves each. The local lesion counts/Xanthi leaf for each treatment were multiplied by the factor by which the sap was diluted. The amount of local lesions in presence of the antimetabolites was compared to that of the control discs.

Test on discs of Xanthi tobacco leaves treated with antimetabolites before TMV inoculation. Leaves selected at random from plants in the five-leaf stage were stacked in tens and discs of 2.5 cm. cut out. Twenty-five randomly selected discs were put, leaf surface up, into glass Petri dishes of 13.5 cm. diameter containing 50 ml. Vickery solution with 3, 30 or 300 μg. actinomycin/ml or 50, 500 or 5000 μg. chloramphenicol/ml. As controls, discs were placed into Vickery solution only. All plates were incubated at 23 ± 1° and 3500 lux for 0, 5, 10, 20, 30, 48 or 72 hr. At each of these time intervals two Petri dishes with antimetabolite and two control dishes were removed, the leaf discs alternately blotted and rinsed with water 3 times. The blotted discs were dusted with carborundum, inoculated with TMV, rinsed with water, transferred to plates containing 50 ml. Vickery solution without antimetabolite and then replaced in the incubation chamber until lesions developed. Lesion counts of antimetabolite-treated discs were compared with those of controls.

Test on discs of Xanthi tobacco leaves treated with antimetabolites after TMV inoculation. This test was conducted in parallel with the test described in the previous paragraph with the following differences: discs were cut out from inoculated leaves, transferred within 3 min. to Vickery solution only and incubated for 0, ½, 1, 2½, 5, 10, 20 or 30 hr. At each of these times two Petri dishes were removed and the blotted and rinsed discs placed in Vickery solution containing actinomycin or chloramphenicol until lesions could be counted. The control discs remained in Vickery solution without antimetabolites until lesions developed. Lesion counts of treated discs were compared to lesion counts of controls.

RESULTS

Calculation of the efficiency factor

To compare the results of all experiments, for each experiment the average number of lesions/leaf or disc obtained in the presence of the antimetabolite was divided by the average number of lesions/leaf or disc in the control. This quotient is called the efficiency factor. A value greater than 1 indicates stimulation, a value less than 1 inhibition. The mean value, M, of the efficiency factors was calculated from several experiments of the same type. For the determination of the 99.73 % confidence interval at first,$$
s_{m} = \sigma/\sqrt{n}$$

was calculated, where $s_{m}$ = standard deviation of the mean, $\sigma$ = standard deviation, and $n$ = number of experiments. For high numbers of $n$, the confidence interval of the mean (99.73 %) is given by $M \pm 3s_{m}$, for lower numbers a greater factor than 3 was
used according to Koller (1953). In figs. 2 to 4 the mean of efficiency factors of each treatment is plotted against incubation time.

**Effect of simultaneous application of TMV and antimetabolites on virus synthesis**

To study the effect of the antimetabolite on the rate of virus synthesis, Samsun plants were inoculated with TMV. Immediately after infection discs were cut out and put into Petri dishes containing a solution of the antimetabolite. At intervals between 30 and 96 hr after infection 40 antimetabolite-treated and 40 control discs were assayed for virus activity as determined by local lesion test on tobacco var. *Xanthi*

![Graph](image)

**Fig. 1.** Effect of actinomycin C on the synthesis of TMV in discs of Samsun tobacco leaves when applied simultaneously with the virus. *Xanthi* leaves had been inoculated with extracts of 2 x 25 actinomycin-treated or control Samsun discs. Each point represents the mean lesion count/leaf obtained from lesion counts of 2 x 16 *Xanthi* leaves. ○, TMV control; ●, 30 μg./ml.; ■, 150 μg./ml.; ▲, 300 μg./ml.

*necroticum.* In the absence of the antimetabolite a steep increase in virus activity was usually observed starting about 48 hr after infection (Fig. 1). When the biosynthesis was accelerated by the antimetabolite the initial virus activity should have been greater

![Graph](image)

**Fig. 2** Effect of actinomycin, chloramphenicol and 8-azaguanine on the TMV in discs of Samsun tobacco leaves. a, actinomycin C; b, chloramphenicol; c, 8-azaguanine. The antimetabolites were applied simultaneously with the virus. The efficiency factors for each concentration and incubation time were calculated (see Methods) from several experiments. In each of six experiments with actinomycin 50 infected discs and 50 controls were assayed, i.e. a total of 600 discs for each point. For chloramphenicol the efficiency factors were calculated from four experiments, i.e. a total of 400 discs/point and for 8-azaguanine from three experiments with 300 discs/point.
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Fig. 2. For legend see facing page.
than in the control, resulting in an efficiency factor (lesions/leaf + antimetabolite): (lesions/leaf - antimetabolite) greater than 1. When virus synthesis was retarded or blocked, the efficiency factor should have been less than 1.

Actinomycin, 1 µg./ml., had no significant effect, whereas concentrations from 10 to 30 µg./ml. led to a two- to fourfold increase of the virus activity 30 hr or more after infection. Later on, the rate of virus synthesis approached that of the control. Concentrations of actinomycin of 150 µg./ml. and greater inhibited virus synthesis. Efficiency factors of 0.01 to 0.004 were reached corresponding to a 100- to 4000-fold inhibition. Similar experiments were done with chloramphenicol and 8-azaguanine (Fig. 2a-c).

Table 2. Maximum increase (efficiency factor) in tobacco mosaic virus synthesis by treatment of tobacco leaf discs with antimetabolites.

<table>
<thead>
<tr>
<th>Antimetabolite</th>
<th>Conc. (µg./ml.)</th>
<th>Efficiency factor</th>
<th>Hr after infection</th>
<th>Conc. (µg./ml.)</th>
<th>Efficiency factor</th>
<th>Hr of pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin</td>
<td>10</td>
<td>2.0</td>
<td>30</td>
<td>3</td>
<td>2.30</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4.75</td>
<td>30</td>
<td>30</td>
<td>2.75</td>
<td>30</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10</td>
<td>4.5</td>
<td>48</td>
<td>50</td>
<td>2.2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>3.6</td>
<td>48</td>
<td>500</td>
<td>3.5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>1.75</td>
<td>48</td>
<td>5000</td>
<td>3.3</td>
<td>20</td>
</tr>
<tr>
<td>8-Azaguanine</td>
<td>0.2</td>
<td>3.25</td>
<td>72</td>
<td>5</td>
<td>4.7</td>
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<tr>
<td></td>
<td>2.0</td>
<td>2.7</td>
<td>72</td>
<td>10</td>
<td>9.8</td>
<td>30</td>
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<tr>
<td></td>
<td>20.0</td>
<td>1.7</td>
<td>72</td>
<td>100</td>
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<td>DL-Parafuor-</td>
<td>5.0</td>
<td>4.7</td>
<td>30</td>
<td>0.2</td>
<td>2.3</td>
<td>48</td>
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<tr>
<td>phenylalanine</td>
<td>10.0</td>
<td>9.8</td>
<td>30</td>
<td>10.0</td>
<td>10.3</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>7.4</td>
<td>72</td>
<td>50</td>
<td>8.3</td>
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<tr>
<td>Puromycin</td>
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<td>50</td>
<td>8.3</td>
<td>30</td>
</tr>
</tbody>
</table>

Biosynthesis was also stimulated by low concentration of DL-parafuorphenylalanine and puromycin as well as the other three antimetabolites used when applied immediately after inoculation of virus (Table 2). Concentrations of 1000 µg./ml. DL-parafuorphenylalanine and 100, 200 and 500 µg./ml. puromycin, however, inhibited the virus synthesis within 96 hr from ten- to 1000-fold.

Effect of treatment with antimetabolites before application of TMV

Pretreatment of leaf discs with actinomycin or chloramphenicol also led to a stimulation (Fig. 3a, b) and to an appearance of lesions from 10 to 21 hr earlier as in the controls. In this type of experiment the discs were cut from tobacco var. Xanthi necroticum and the number of lesions counted directly.

Effect of treatment with antimetabolites after application of TMV

When actinomycin and chloramphenicol were added to infected leaf discs of tobacco var. Xanthi necroticum from ½ to 30 hr after inoculation the virus synthesis was not significantly affected (Fig. 4a, b). The other antimetabolites were not investigated in this and the previous test.
Discussion

Although we used different tobacco species and other methods, our results with antimetabolites are in agreement with some experiments of Prozorovskaya, Balandin & Tongur (1966), who observed a stimulation of TMV synthesis in leaves of *Nicotiana glutinosa* by pretreatment with 2.5 μg. chloramphenicol/ml. nutrient solution. In a cell-free system, they noted an increase in replicative form of RNA of 115% over the controls. By their methods, however, 10 μg. actinomycin D/ml. had no influence on TMV synthesis *in vitro*, and as little as 50 μg. puromycin/ml. showed an inhibitory effect. The difference in dosage effect between the antimetabolites used here and those stated in the literature may be explained by different rates of diffusion of antimetabolites into cells of different tobacco species; and also the sensitivity of cell metabolism to different types of antimetabolites is known to vary (Parthier, 1965).

Once established, TMV synthesis was not influenced significantly by the addition
of any concentration of actinomycin C and chloramphenicol in our experiments. This suggests a blocking effect by the antimetabolites on a factor(s) otherwise interfering with virus synthesis. Such a factor has been isolated from virus-infected animal systems as interferon (Isaacs, 1963; Marcus & Salb, 1966). According to Levine (1964) the inhibitory effect of interferon on multiplication of western equine encephalitis virus could be reduced when cells were treated at the same time with interferon and either puromycin-hydrochloride or actinomycin D as well as when they were treated with actinomycin D 1 to 4 hr before giving interferon. From these experiments, it is concluded,

Knowing actinomycin as inhibitor of DNA specified RNA synthesis and puromycin as inhibitor of protein synthesis, that RNA and protein must be synthesized by cells treated with interferon before interferon can inhibit virus multiplication.

Results obtained by Ross (1966), Sela, Harpaz & Birk (1966), and Loebenstein, Shlomit & van Praagh (1966) led to the conclusion that there is also a virus-inhibiting factor or factors in plants when leaves are inoculated with TMV. From these experiments it cannot be decided, however, whether the factor is newly synthesized or merely activated by TMV infection. Our results are in agreement with the concept of the occurrence of a virus inhibiting factor in plant tissue.
Stimulation of TMV biosynthesis by antimetabolites

As Röttger (1965) measured a marked increase in leaf RNA after inoculation of tobacco leaves with TMV, the stimulation of TMV biosynthesis by antimetabolites may be thought of as a consequence of their suppressing virus-induced synthesis of leaf-RNA and -protein necessary for the formation of antivirus factor; with antiviral action impaired, TMV biosynthesis can proceed unhindered and therefore appears as stimulated. Our results obtained by the treatment of leaf discs with antimetabolites immediately after virus inoculation are in accordance with this hypothesis. Results obtained by treatment of leaf discs with antimetabolite before virus inoculation, however, suggest also the possibility of a factor with antiviral properties already present in plant tissue becoming activated upon virus infection, but the activation process or the activated factor itself can be blocked by antimetabolites permitting then the unhindered biosynthesis of TMV.

Fig. 4b

Fig. 4. Effect of treatment after inoculation with (a) actinomycin C and (b) chloramphenicol on TMV synthesis in discs of Xanthi tobacco leaves. From five experiments/antimetabolite the efficiency factors for each concentration and time of antimetabolite application after inoculation were calculated (see Methods). Each point represents lesion counts on 40 treated discs and 40 controls, i.e. on a total of 400 discs.
The inhibition of TMV biosynthesis by higher concentrations of the antimetabolites can be attributed to an impairment of the cell metabolism as a whole since leaf discs began to become discoloured at 96 hr after inoculation whereas the discs in lower concentrations did not.

The lack of an effect on the extent of virus biosynthesis when actinomycin and chloramphenicol were applied ½ to 30 hr after inoculation indicates that the activation or formation of the factor (or factors) and its action must be an event occurring within the first 30 min. after inoculation of plant tissue.

The author wishes to thank Professor Schramm for valuable discussions during the conduct of this investigation. The skilful technical assistance of Miss L. Ostendorff, Miss M. Klein and Miss A. Kleih is gratefully acknowledged. For the gift of samples of actinomycin C and chloramphenicol thanks are due to the Farbenfabriken Bayer, Leverkusen, Germany. The work was supported by the U.S. Public Health Service (grant no. CA 04601-08).

REFERENCES


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