ACTION OF ACTINOMYCIN D ON RNA SYNTHESIS IN HEALTHY AND VIRUS-INFECTED TOBACCO LEAVES*

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Actinomycin is known to inhibit specifically DNA-dependent RNA synthesis in mammalian and bacterial cells, presumably by combining with the DNA template (Reich et al., 1962) and by preventing the action of the DNA-dependent RNA polymerase (Hurwitz et al., 1962). A similar situation appears to apply to plant cells as shown recently by Click and Hackett (1963) with slices of potato tubers. In all of these cases the inhibition applies to nuclear, ribosomal, and transfer RNA, but the synthesis of transfer RNA is inhibited somewhat less than the other types (Tamaoki and Mueller, 1962; Merits, 1963).

In the case of viral infections, actinomycin has been shown to inhibit the development of DNA viruses if it is applied prior to or simultaneously with infection, but it has little effect on the reproduction of RNA-containing animal viruses (Reich et al., 1961, 1962; Reich and Franklin, 1961; Franklin and Baltimore, 1962).

Evidence is presented here that actinomycin D inhibits the incorporation of uracil-2-^{14}C in all forms of RNA in normal tobacco leaves, but in mosaic-diseased tobacco leaves treated in the same manner, uracil-2-^{14}C is incorporated into the 28 S RNA fraction. These results indicate that a DNA template is not involved in the synthesis of tobacco mosaic virus RNA.

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MATERIAL AND METHODS

Intact leaves of Nicotiana tabacum L. var. Turkish, either healthy or infected with RNA of the common strain of TMV, were used for incorporation studies. The TMV-RNA was freshly prepared by the phenol method (Gierer and Schramm, 1956) shortly before inoculation at a concentration of 50 μg/ml. Labeling of RNA of the cells was accomplished by allowing individual leaves to take up through the petiole 2 μc uracil-2-^{14}C (Sp. act. 6.5 mc/mmole) in 0.5 ml. of water. The leaves were held in 1 ml. of water, kept in a water-saturated atmosphere, and were illuminated during the incorporation period, which was terminated by the RNA extraction. In the inhibition studies, 1-50 μg. of actinomycin D were added per leaf and also allowed to be taken up through the petiole. The RNA of the tobacco leaves was extracted and fractionated on sucrose density gradients according to the following newly devised procedure:

Ten grams of plant material were thoroughly homogenized in an ice-chilled Servall Omni-Mixer, run at low speed, in the presence of 20 ml. of water-saturated phenol and 10 ml. of glycine buffer (0.1 M glycine, 0.1 M NaCl, 0.005 M EDTA, adjusted to pH 9.5) containing 1% bentonite. The emulsion was broken by centrifugation. The aqueous supernatant was saved and the lower phenol phase discarded. The interphase consisting of a green, homogeneous slurry was homogenized twice more with 20 ml. phenol and 10 ml. bentonite-containing buffer. The two resulting supernatants were pooled with the first and the combined aqueous phases were stirred for 15 minutes with 0.1% bentonite and with an equal volume of phenol. After centrifugation the aqueous layer was carefully removed without disturbing the interphase and stirred once more with 0.1% bentonite and an equal volume of phenol. The aqueous layer was then shaken four times with two volumes of ether to remove the phenol, and the ether was removed by bubbling nitrogen through the solution. The RNA solution was next desalted (large amounts of salt from the plant tissues are carried through the phenol extractions in the aqueous phase) by passage through a column containing five times its volume

of Sephadex G25. As the effluent emerged from the column, fractions were collected and their absorbancies were read at 260 mμ. It was found that the RNA appears immediately after the void volume while salts and certain colored substances are retained by the Sephadex. The RNA fractions were pooled, and the RNA was twice precipitated with two volumes of cold ethanol and resuspended in water or glycine buffer.* The fractionation of the RNA was carried out in 5-20% sucrose gradients prepared with glycine buffer. One ml. of a solution containing 2-3 mg. of RNA was layered on top of the sucrose gradient** and was centrifuged in the SW 25 Spinco rotor for 16 hours at 24,000 rpm and 5-10° C. Approximately 30-40 fractions were collected at the end of the run through a hole pierced in the bottom of the tube, and the absorbancies of the fractions were read at 260 mμ after suitable dilution. After measurement of the total absorbancy at 260 mμ, 0.1 ml. portions of the successive fractions were dissolved in 10 ml. scintillation fluid and the radioactivity was counted in a Packard automatic Tri-Carb liquid scintillation counter.

The infectivity of the TMV or of its RNA was tested in the usual way on leaves of Nicotiana tabacum L. var. Xanthi-nc and infectivities are expressed in average numbers of lesions per leaf using 6 leaves per sample.

RESULTS

The yields of RNA extracted by the above procedure ranged between 0.5 and 0.8 mg. per gram of leaf tissue, assuming that 1 mg. of RNA per ml.

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* The fine, flocculent, highly soluble precipitates obtained from solutions desalted with Sephadex are in sharp contrast to the coarse, granular, poorly soluble precipitates of non-desalted solutions. Furthermore, the RNA precipitated from desalted solutions is found, as judged by sedimentation behavior, to retain its physical integrity for several months when stored in glycine buffer or in the frozen state.

** Traces of ethanol in the RNA solution cause turbulence by mixing with the sucrose when layered on top of the gradient solution. Special care was taken to remove these traces by evaporating them with nitrogen from the ethanol precipitate and by bubbling nitrogen through the final solution. (A helpful step suggested by Mr. R. E. Click is to sediment the last ethanol precipitate of RNA, pour off the supernatant fluid and then centrifuge the precipitate for an additional time to pack it tightly and permit removal of more alcoholic solution. The precipitate is then dissolved and treated with nitrogen as above.)

has an absorbancy of 25.0 at 260 mμ. The RNA contained less than 1% of DNA and protein as determined by colorimetric tests and gave a typical absorption spectrum in the ultraviolet with a 258/230 mμ ratio of 2.4-2.8. The bulk of the RNA extracted from normal tobacco leaves was fractionated on a sucrose density gradient into three components which upon removal and examination in the ultracentrifuge in glycine buffer at pH 9.5 were found to have sedimentation coefficients of 28S, 17S, and 4S (Fig. 1). The RNA of tobacco leaf ribosomes appeared to contain mainly 28S and 17S components, whereas all three components were found in the RNA extracted from tobacco leaves infected with TMV. Infectivity, however, was associated only with the 28S material (Fig. 2).

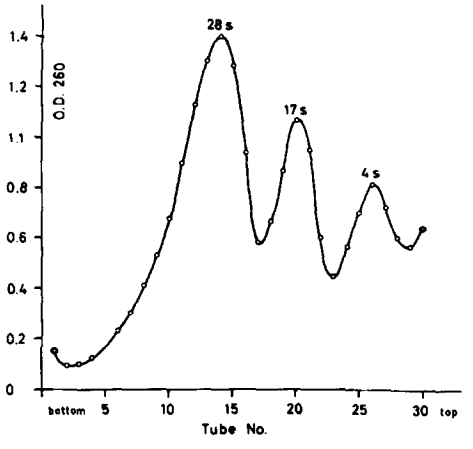


Fig. 1. Sedimentation profile of RNA from tobacco leaves in a 5- 20% sucrose density gradient.

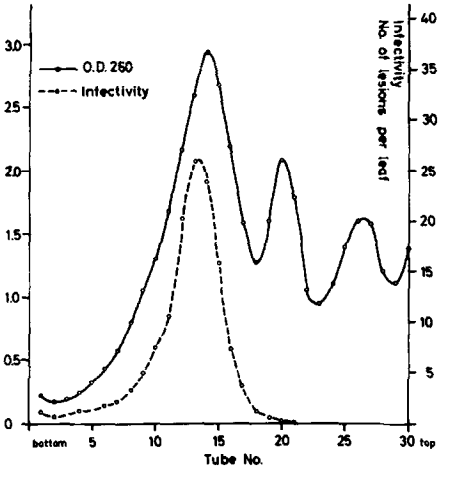
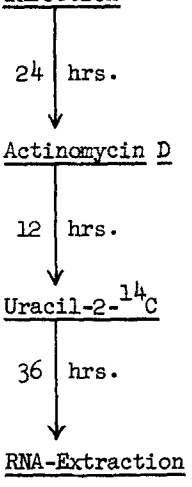


Fig. 2. Sedimentation profile of RNA from leaves infected with tobacco mosaic virus. (Infectivity = average from 6 leaves)

By 14-36 hours after the uptake of uracil-2-^{14}C into tobacco leaves, radioactivity was found in all of the RNA, and radioactivity and UV absorption were nearly parallel (Fig. 3). A very similar pattern was obtained if the leaves were infected with TMV-RNA 18-36 hours prior to the uptake of radioactivity. When all successive fractions were tested for infective TMV-RNA, infectivity was found under the 28 S peak (Fig. 4). In healthy leaves the incorporation of uracil-2-^{14}C into cellular RNA was

markedly suppressed if actinomycin D was taken up prior to the incorporation. There was, however, some incorporation even with high levels of actinomycin D if it was applied simultaneously with the uracil-2-^{14}C. Twenty-five µg. of actinomycin D per leaf applied 12 hours before the uptake of radioactivity produced 95% inhibition of the incorporation of uracil-2-^{14}C into the 28 S and 17 S RNA through a 24-36 hours incubation period. In accordance with the results obtained in other systems (Tamaoki and Mueller, 1962; Merits, 1963), the incorporation into the 4 S RNA fraction was inhibited to a lesser extent (Fig. 5). If, however, under similar experimental conditions, leaves were used which had been infected with TMV, considerable labeling did occur in the 28 S RNA fractions. The results of a typical experiment set up according to the following scheme are shown in Fig. 6.



Bioassay of all fractions showed that infective TMV-RNA is synthesized under conditions where the synthesis of cellular RNA is almost completely inhibited. Infectivity and radioactivity were very nearly parallel and appeared, like TMV-RNA, under the 28 S RNA peak. Buffer homogenates of tissue samples also showed clearly that the synthesis of complete TMV is carried out under these conditions, but the yield of infective virus appears to be about 60-80% of the yield of the untreated controls.

Consonant with the results obtained in animal and bacterial systems, the present findings support the concept that cellular RNA synthesis is also DNA dependent in plants. However, in the TMV system, the synthesis of viral RNA seems to occur without the benefit of a DNA template. The

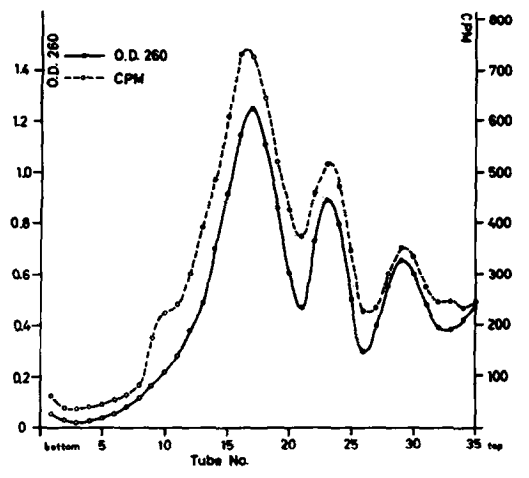


Fig. 3. Sedimentation profile of tobacco leaf RNA labeled by 36 hours exposure to uracil-2-^{14}C.

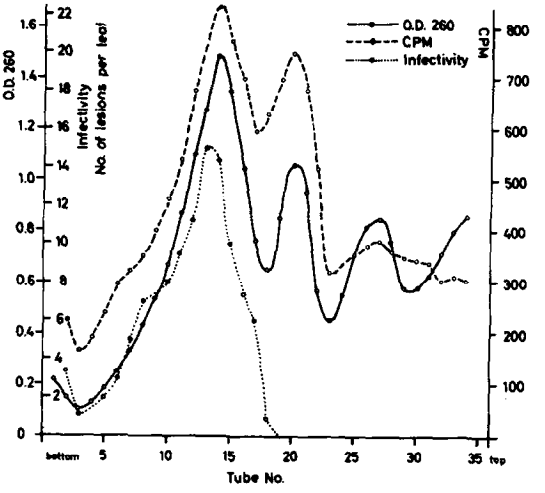


Fig. 4. RNA from leaves infected with TMV-RNA 36 hours before the 36 hours labeling.

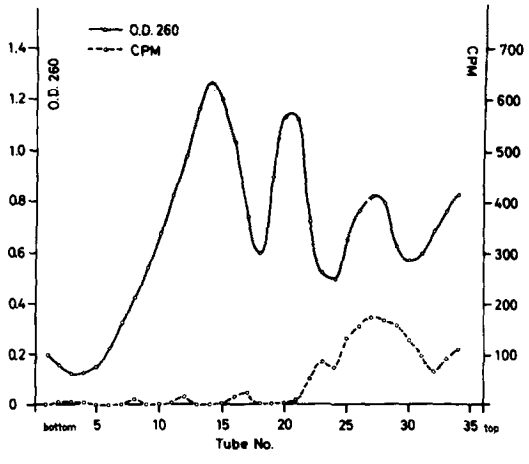


Fig. 5. Inhibition of incorporation of uracil-2-^{14}C into tobacco leaf RNA by 25 μg actinomycin D per leaf.

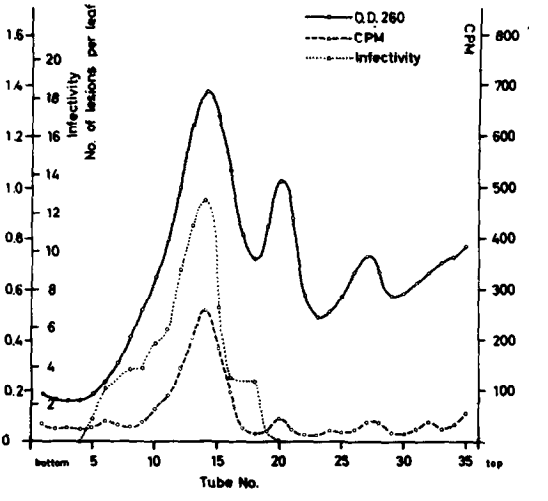


Fig. 6. Incorporation of uracil-2-^{14}C into 28 S RNA under conditions of Fig. 3 except that leaves have been infected with TMV.

same conclusion was recently reached by somewhat indirect experiments based on a comparison of DNA from healthy and infected plants (Reddi and Anjaneyalu, 1963).

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