NATURE OF INFECTIOUS MATERIAL IN DEOXYRIBONUCLEOPROTEIN PREPARATIONS FROM LEAVES INFECTED WITH TOBACCO MOSAIC VIRUS

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The synthesis of infectious tobacco mosaic virus (TMV) ribonucleic acid (RNA) in cell-free systems prepared from healthy or virus-infected leaves has been claimed in several recent reports (Kim and Wildman, 1962; Cochran et al, 1962; Karasek and Schramm, 1962; Bandurski and Maheshwari, 1962). Using a method proposed by Hudson, Kim and Wildman (1963) for the preparation of deoxyribonucleoprotein (DNP) from tobacco leaves we have been unable to obtain convincing evidence for *in vitro* synthesis of infectious material.

Frozen leaf was ground with twice its weight of water, 1% weight of bentonite and sand. After centrifuging at 15,000 r.p.m. for 20 mins the resulting pellet was ground again in 0.1M glycine, 0.05M K_2HPO_4 adjusted to pH 9.5 plus 20% NaCl. After centrifuging at 7,000 r.p.m. for 10 mins two volumes of ethanol were added to the supernatant solution. After 30 mins at 2° the floating DNA was removed, resuspended in buffer, clarified by centrifugation and reprecipitated with ethanol. The yields of DNP varied considerably. Expanded leaves with a dry weight/fresh weight ratio of about 0.15 were found most

suitable, yielding about 16 mg. of dry DNP from 50 gms. fresh weight of leaf. About 5 mg. of DNA was recovered from such a sample of DNP by phenol extraction (Kirby, 1957). In a typical assay for the synthesis of infectious material the DNP from 50 gms. of infected leaf was dissolved in 5 ml of 0.14M NaCl at 4°. Aliquots (1 ml) of this solution were added to a series of reaction mixtures containing 0.6 ml of the second medium of Kim and Wildman (1962) and 0.6 ml of a solution of the four nucleoside triphosphates (each 1μM/ml). The solutions were kept in ice during preparation and were incubated according to the chosen conditions of the experiment. They were then returned to ice and inoculated to N. glutinosa as soon as possible using a replicated latin square design.

Confirming Kim and Wildman (1962) we obtained a rise in lesion number following incubation at 25° (e.g. Table 1, samples A and B).

TABLE 1

Infectivity of mixtures containing DNP from TMV infected tobacco leaves and nucleoside triphosphates, after incubation under various conditions.

Sample	Time of Incubation	Temperature	Ribonuclease	Lesions on 15 leaves
A	0	4	-	118
B	60	25	-	217
C	5	60	-	394
D	5 followed by	60	-	-
	60	25	-	361
E	5 followed by	60	-	-
	15	37	10 μg	2

However an even greater amount of infectious material was found after heating the solution at 60° for 5 mins (sample C). No additional rise occurred on incubating heated material at 25°

for 60 mins (sample D). The infectious material was sensitive to ribonuclease (sample E). In other experiments the increase in infectivity following incubation at 25° was independent of actinomycin D (1.0 μg/ml) suggesting that DNA dependent RNA synthesis was not involved in the increase (Hurwitz, Furth, Malamy and Alexander, 1962).

At 60° in the standard medium optimal release of infectious material was complete in one minute or less. (Total lesions on 18 leaves were:- 0 mins at 4°, 16; 1 min. at 60°, 207; 3 mins at 60°, 175; 6 mins at 60°, 93; 9 mins at 60°, 59; 12 mins at 60°, 4.) The optimal temperature for release of infectivity after 5 minutes heating was 50°-55°. (Total lesions on 18 leaves were:- 28°, 2797; 40°, 3559; 45°, 4017; 50°, 5412; 60°, 2329; 70°, 779.) Decrease in infectivity at high temperatures was presumably due to ribonuclease action and/or heat inactivation.

In tests with DNP made from leaves inoculated with infectious TMV RNA we obtained zero infectivity from leaves harvested after 10 hours. With leaves infected for 20 hours the situation was similar to that found with fully infected leaf but with lower lesion numbers.

Since Schramm and Zillig (1955) incubated TMV near 0° at pH 10-10.5 to prepare TMV protein it seemed that the pH of 9.5 used to isolate DNP might expose the RNA of intact virus. On suspending TMV (150 mgm) in 20% sodium chloride and glycine buffer at pH 9.5 for 20 minutes at 4° and precipitating with 2 volumes of ethanol we obtained a heavy precipitate which was centrifuged off. This pellet, when extracted with water or ribonuclease, gave a clear supernatant solution with a typical RNA spectrum when clarified at 2,000 r.p.m. Thus during the DNP isolation the TMV is partly or wholly stripped of its protein coat and becomes ribonuclease sensitive.

Tests showed that such altered TMV can contaminate the DNP. For example DNP was prepared by the standard two precipitations from 50 gms of healthy leaf mixed with 150 mg of TMV, and was then incubated in the standard medium and assayed. Lesion numbers (15 leaves) were:- zero time with triphosphates at 4°, 22; 2 mins at 55° with triphosphates, 80; 60 mins at 25° with triphosphates, 9; 60 minutes at 25° no triphosphates, 31. A sample of the healthy leaf crushed and rubbed undiluted on 15 leaves gave no lesions.

In the experiment summarised in Table 2, DNP isolated from infected leaf was assayed in medium with triphosphates after each of three successive precipitations with 2 volumes of ethanol at pH 9.5.

We interpret our results as follows. The method used to isolate DNP alters intact TMV to make it sensitive to ribonuclease. Variable amounts of this material are carried over with the DNP during ethanol precipitation, either occluded to the DNP or precipitated independently. Different levels of contamination explain the wide variation in infectivity of

TABLE 2

Infectivity of DNP from TMV infected leaves after each of three successive precipitations with ethanol.

Precipitate	Time of Incubation (mins.)	Temperature	Ribonuclease	Lesions on 18 leaves
First	0	4	-	4017
"	2	55	-	1967
Second	0	4	-	457
"	2	55	-	219
"	10	37	10 µg	0
Third	0	4	-	11
"	2	55	-	216
"	10	37	10 µg	0

the DNP preparations at zero time found in our data and that of Kim and Wildman (1962). Appropriate heating or incubation disperses the occluded material giving an apparent increase in the amount of infectious material present. At the same time thermal inactivation and probably ribonuclease action is tending to reduce infectivity.

To test whether new polynucleotides are synthesised during incubation of the DNP with the nucleoside triphosphates, adenosine triphosphate uniformly labelled with P^{32} was included in the standard incubation mixture containing 2.5 times the usual amount of DNP. After incubation carrier TMV RNA was added. The precipitated RNA was hydrolysed with alkali and the mixed nucleoside 2' and 3' phosphates were isolated. A low level of radioactivity was found in the mixed adenosine, cytidine and uridine 2' and 3' phosphates. This radioactivity might have been due to terminal addition of nucleotides to soluble RNA, since the guanosine 2' and 3' phosphates were not labelled. If the radioactivity present was the result of polynucleotide synthesis it represented 1.0 μg of RNA in the 2.2 ml mixture. Had this been newly formed infectious TMV-RNA with the same specific infectivity as RNA made by phenol from whole virus, it would, under our conditions, have increased lesion numbers by approximately 0.5-2.0 per leaf. Proof of TMV-RNA synthesis would be difficult in a system contaminated with ribonuclease and with a large and variable amount of ribonuclease-sensitive infectious material.

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