A RIBONUCLEOTIDE POLYMERASE FROM TOBACCO LEAVES AND THE FORMATION OF VIRAL RIBONUCLEIC ACID IN VITRO

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The formation of infectious ribonucleic acid during the multiplication of tobacco mosaic virus (TMV) has been described in vivo (Engler and Schramm,1960). In vitro, the synthesis of RNA in crude extracts of tobacco plants is difficult to observe because of the relatively high content of nucleases released during the extraction of plant enzymes. By use of the absorbant bentonite (Brownhill, Jones, and Stacey, 1959) it has been possible to study the incorporation of ATP into acid insoluble polyribonucleotides and to concentrate the responsible enzyme. In addition to catalyzing the polymerisation the isolated protein fraction increases the infectivity of RNA molecules in cell-free extracts from tobacco plants infected with TMV. The relation between the RNA polymerase of tobacco leaves and the formation of infectious RNA in vitro is discussed in the present paper.

Methods: Leaves from normal or infected (3-14 days prior to the experiment)young tobacco plants (var. Samsun) were employed. The mid veins were removed, and the tissue homogenized in an ice-cold buffer (Buffer A) that contained 0.01 M Tris, pH 7.4 , 0.05 M KCl, 0.001 M $MgCl_2$, 0.0001 M EDTA, and 0.0001 M dimercaptopropanol. The pH was adjusted to 7.2 and the broker

cells were removed by a preliminary centrifugation for 15 minutes at 15,000 RMP (Spinco Model L). The supernatant was treated for 15 minutes at 0° with a suspension of bentonite (200 mg/ml) corresponding to 10 percent of the total volume of the supernatant. The bentonite had been previously washed with Buffer A and suspended in distilled H_2O to provide a concentration of 200 mg/ml. The suspension was centrifuged for 1 hour at 40,000 RPM to sediment bentonite and intact virus particles present in infected tissue. 1.0 volume of saturated ammonium sulfate, pH 7.0, previously recrystallized in the presence of 0.001 M EDTA, was added with stirring, the precipitate allowed to form for 15 minutes, and centrifuged for 15 minutes at 15,000 RPM. The tubes were drained and the precipitate dissolved in Buffer A to provide a concentration of 30 mg protein/ml.

The enzymatic activity and infectivity were determined following an incubation of 2 hours at 30° in a reaction mixture that contained the following components in a total volume of 0.5 ml: 10 µmoles Tris buffer, pH 7.6, 0.25 µmoles $MnCl_2$, 1.0 µmoles $MgCl_2$, 3.0 µmoles dimercaptopropanol, 5.0 mµmoles EDTA, 3.0 mg enzyme protein, 1.0 mg bentonite, and 100 mµmoles each of CTP, GTP, UTP, and ATP-8-C^{14} containing 500 cpm/mµmole. To determine the incorporation of ATP the reaction mixture was chilled and 1.0 mg each of carrier yeast RNA and serum albumin (10 mg/ml) was added. The proteins and nucleic acids were precipitated with 5.0 ml of cold 3.5% perchloric acid. The precipitate was dissolved in 1.0 ml concentrated formic acid, centrifuged to remove the bentonite, plated on aluminium planchets, dried, and counted in a windowless gas flow counter. To determine infectivity aliquots of the reaction mixture were diluted with distilled water that contained

0.1 mg bentonite/ml and assayed on either Nicotiana tabacum var.Xanthi or Nicotiana glutinosa by standard assay procedures employed in this laboratory (Engler and Schramm, 1960).

Results: The first ammonium sulfate precipitate containing the polymerase has an optical density ratio of 1.14 280/260 indicating the presence of nucleic acid. In the enzyme isolated from infected plants part of the associated nucleic acid consists of infectious TMV-RNA since the infectivity of this material is destroyed rapidly by ribonuclease. The enzymatic activity of this fraction is illustrated in Table 1.

Table I

Requirements for Incorporation of ATP into Polynucleotides

System	Omission	mμmoles ATP incorporated
complete	-	11.2
"	GTP,CTP,UTP	9.8
"	bentonite	0.27
"	bentonite and GTP,CTP,UTP	0.01
"	Enzyme	0.36

The incorporation of ATP is maximal in the presence of nucleotide triphosphates and bentonite. Optimal incorporation is observed at 2 hours at pH 7.0. Addition of bentonite leads to a 40 fold increase of C^{14} in the acid insoluble precipitate. The effect is also observed when bentonite is added after the incubation showing that the clay has an effect on the precipitation as well as on the formation of the reaction product. The C^{14}-containing material is stable to deoxyribonuclease, partially hydrolyzed by ribonuclease, and completely hydro-

lyzed by 0.5 N KOH at 37° for 18 hours suggesting that the C^{14} adenine is incorporated into a polyribonucleotide. For further characterization the reaction mixture has been separated on a 150 x 1 cm column of Sephadex G 25. Fig.1 shows that several distinct peaks appear in the eluate. The main radioactivity is associated with peak II which has a sedimentation constant of 1-1.5 S and an estimated molecular weight of about 10,000 - 20,000. 0.18 percent of the total radioactivity is associated with a high molecular weight product which emerges with the peak I of infectious TMV-RNA.

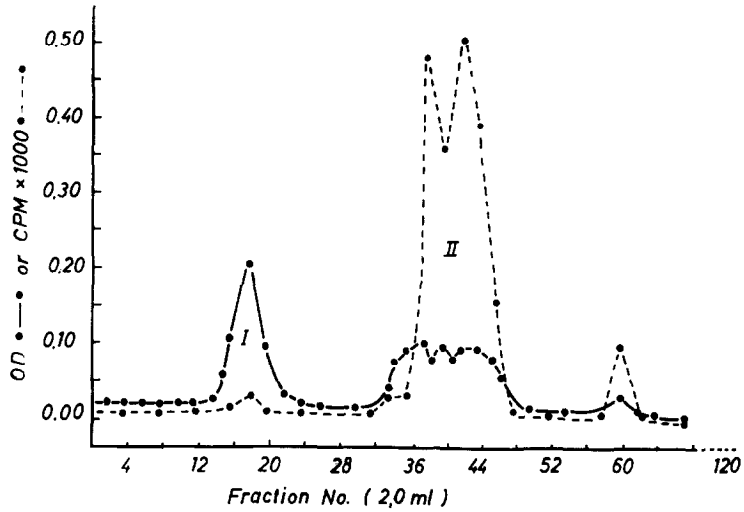

Fig.1: Separation of labeled polynucleotides on Sephadex G 25

The distribution of the peaks and the radioactivity is similar in normal and infected tissues, but different effects on added infectious TMV-RNA have been observed. Incubating TMV-RNA with the enzyme from normal tissue the infectivity is destroyed in 30 minutes; in contrast, the enzyme preparation from infected plants either increases the infectivity after

a 2 hour incubation (8 experiments) or prevents the destruction of infectivity (3 experiments). A representative example is shown in Table II.

Table II

Requirements for Formation of TMV-RNA

System	time (hours)	Lesions
complete	0	574
"	1	694
"	2	718
"	4	614
"	8	290
- nucleotides	2	619
+ RNase	2	0
+ DNase	2	439

The increase in activity is time-dependent and sensitive to ribonuclease. A weak inhibition of infectivity has been observed with deoxyribonuclease and by omission of nucleotides.

Discussion: An enzyme can be detected in tobacco leaves which leads to the incorporation of ATP into an acid insoluble material containing oligonucleotides and high molecular RNA. The activity of this enzyme is nearly the same in normal and infected tissues. Only a partially purified enzyme has been studied and the nature of any necessary primer has not been determined. Enzyme preparations from normal tissue destroy the infectivity of added TMV-RNA probably due to a contamination with ribonuclease. Identical preparations from in-

fected tissues increase the activity of the viral RNA showing that a process is induced that predominates over the destruction of RNA by ribonuclease. This process can be interpreted as a release of infectious RNA from unknown sources by a time and energy dependent reaction, as a net synthesis of infectious RNA molecules, or as a completion of preexisting partially synthesized RNA. The small amount of radioactivity incorporated into the fraction containing the infectious RNA and the failure to increase the infectivity in normal tissues which do not contain precursors of infectious nucleic acid is in favor of the latter interpretation.

References

1. Brownhill, T.J., Jones, A.S., and Stacey, M. Biochem.J. 73, 434 (1959)
2. Engler, R., and Schramm, G. Zeitschrift für Naturforschung 15b, 38 (1960)