Reactivation of tobacco mosaic virus infectivity in mixtures of virus protein and nucleic acid*

The possibility of reactivating non-infectious fragments of tobacco mosaic virus (TMV) has held considerable interest for investigators. In 1947 SCHRAMM[1] showed that a mild alkaline treatment would dissociate TMV nucleoprotein and yield undenatured low-molecular weight non-infectious protein fragments which were readily repolymerized to form particles resembling the virus in size. SCHRAMM studied the infectivity of material from *in vitro* mixtures of this dissociated protein (A-protein) with preparations of TMV nucleic acid. In 2 out of 15 instances he obtained a significant increase in infectivity (as determined by local lesion counts) of the protein-RNA mixture over the residual infectivity of the unmixed starting materials. Because of the variability of the results, SCHRAMM concluded that he had failed to establish the validity of the phenomenon. TAKAHASHI AND ISHII[2] suggested that similar efforts be made to obtain infectious complexes from mixtures of virus RNA and a non-infectious protein which accompanies TMV in infected plants, but have not reported any success. COMMONER *et al.*[3] reported the formation of an artificial nucleoprotein, which proved to be non-infectious, in mixtures of virus RNA and a non-virus protein (B8).

A new approach to this problem is made possible by SCHRAMM's recent report[4] of an improved method for dissociating TMV protein. The present paper reports apparently successful reactivation of mixtures of this protein and virus RNA. FRAENKEL-CONRAT AND WILLIAMS have just reported comparable results[5]. Our work was carried out independently of theirs.

Preparations of dissociated TMV protein were obtained according to the method described by SCHRAMM *et al.*[4]. An aqueous solution of TMV was brought to pH 10.3 with NaOH and after three hours at 2° C, dialyzed against distilled water for 10–16 hours. Residual unbroken TMV was removed from the preparation by two ultracentrifugations at 104,500 × *g*. Nucleic acid was prepared from the same original stock of TMV by a modification of the method of COHEN AND STANLEY[6]. The product was ultracentrifuged twice to remove residual virus and denatured protein and dialyzed against distilled water.

The protein and nucleic acid preparations were then mixed and treated immediately with ammonium sulfate to induce polymerization. This was accomplished by adding solid salt to the mixture until 0.4 saturation was attained. Mixtures were held in this state for 1–6 hour periods at 2° C. The material rendered insoluble by the salting-out process was then removed by centrifugation and redissolved in pH 7 phosphate buffer. High-molecular weight material was then removed by ultracentrifugation (104,500 *g* for 1 hour) and the resulting pellet taken up in a fixed volume of phosphate buffer. This final solution was then used for infectivity tests. In each experiment, aliquots of the separate protein and nucleic acid preparations equal in amount to the material used to prepare experimental mixtures, were set aside for control purposes. In all but the first two experiments, the separate component preparations were treated in a manner identical with the schedule employed with protein-nucleic acid mixtures, including the polymerization step. Hence, the final product yielded by these controls, and tested for infectivity, represents a mixture of (a) residual actual TMV remaining in the separate components after preparation and despite purification steps and (b) any new infectious material formed within the separate component preparations as a result of polymerization. Thus, in a protein preparation treated in this manner, some virus reactivation might be expected to occur because of the possible presence of still active virus nucleic acid. In four experiments, controls were represented by protein-nucleic acid mixtures which were identifical in composition to the mixtures described above, and treated in a like manner, except that the polymerization step was omitted. These controls would be expected to show infectivities resulting from the combined residual TMV contents of the components used to prepare the experimental mixtures.

With the exceptions noted in Table I, the amounts of TMV used to prepare protein and nucleic acid components were in the ratio 2:1. In all cases, the starting material was obtained from a single stock of TMV extensively purified by successive isoelectric and ultracentrifugal precipitations and found to be homogeneous in the Tiselius apparatus.

Infectivity tests were carried out with leaves of *Nicotiana glutinosa*. These were removed from the plants and maintained during the test period with their petioles immersed in water at 24° C and 100 foot candles of constant illumination. Each test preparation was made up to a comparable volume (5 or 10 ml) and applied to 10 or 20 randomized leaves with small gauze pads. Lesions were counted 4 days after inoculation. In each experimental series a comparable set of leaves was inoculated with a standard preparation of ordinary, untreated TMV.

The results of eight sets of experiments are summarized in Table I. In each experiment the protein-nucleic acid mixtures polymerized by means of ammonium sulfate yielded material which contained significantly more infectivity than the controls. The absolute number of lesions

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TABLE I

REACTIVATION OF INFECTIVITY IN MIXTURES OF TMV COMPONENTS

Experiment No.	Starting* material mg TMV	Final volumes of preparations tested ml	Infectivity: total number of lesions obtained**				
			Separate controls		Mixed controls (unpolymerized)	Polymerized mixtures	TMV*** standards
			Protein	Nucleic acid			
1	69.9	10	—	—	6	330	135
2	87.4	10	—	—	19	387	328
3	87.4	10	13	1	15	149	78
4	58.5	10	2	—	2	80	44
5	87.4	10	0	0	—	42	117
6	69.9	10	33	7	—	146	257
7	136.9	5	3	5	—	154	184
8	75.7	5	59	0	—	432	230

* This material was used to prepare protein and nucleic acid components in the ratio 2:1, except for experiment No. 7 in which the ratio was 1:2, and experiment No. 8 where the ratio was 7:8.

** Each control, polymerized preparation and TMV Standard tested on 20 leaves of *N. glutinosa*, with the exception of experiments 1 and 4, in which 10 leaves were employed.

*** TMV Standards were 10 μg/ml in phosphate buffer, except in experiments 6, 7 and 8 in which concentrations of 20 μg/ml were used.

obtained per leaf varies considerably, as do the lesion counts given by the TMV standards. In all cases however, the mixtures gave lesion counts 3 to 10 times greater than the combined infectivities of the control samples. An increase in infectivity of this magnitude found without exception in eight trials cannot be ascribed to chance variations in the response of separate sets of *N. glutinosa* leaves to infection with TMV. We conclude, therefore, that the processes previously described do, in fact, restore to relatively uninfectious preparations of TMV protein and nucleic acid, a significant amount of virus infectivity. As judged from a crude comparison with the TMV standard included in each experimental series, the added infectivity induced by the treatments employed represents that expected from about 40–250 μg of TMV.

One explanation for these results is that the infectivity and by inference the genetic specificity of TMV, is a joint property of its nucleic acid and protein, and that the infectivity regained in artificial mixtures results from specific recombination of the two components into a nucleoprotein identical with the virus. However, it is equally possible that protein or nucleic acid alone carries the virus' biological activity, and that mixture with the second constituent serves only to protect the active material from degradation. Resolution of this question will require the actual isolation and characterization of the particles responsible for the newly activated infectivity of the mixtures. Such an identification of infectivity with a specific particle is not yet possible because only a very small part of the repolymerized protein-nucleic acid mixture is infectious. For this purpose it will become necessary to produce material that approaches actual virus in specific infectivity.

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