Antigenic Study of the Protein from a Defective Strain of Tobacco Mosaic Virus

Abstract. *Soluble protein from the defective strain PM2 of tobacco mosaic virus can be recognized as antigenically distinct from the protein of the wild-type tobacco mosaic virus by Ouchterlony and immunoelectrophoretic analyses at temperatures below 37°C. At these temperatures a polymerized state of protein with a characteristic antigenic structure is present in the wild-type virus; this structure is absent from the PM2 preparations. Above 37°C the precipitin patterns for tobacco mosaic virus and PM2 appear identical.*

A defective mutant strain (PM2) of tobacco mosaic virus (TMV) was first described by Siegel *et al.* (*1*). It was isolated from *Nicotiana tabacum* after treatment of the common strain of TMV (U1) with nitrous acid. The mutant is sensitive to ribonuclease and is not inactivated by purified rabbit antibody directed toward the coat protein of TMV. The coat protein synthesized by the mutant virus remains free in the cytoplasm and is apparently unable to bind with the infectious RNA. As a result, no complete virions are produced, although PM2 protein can polymerize with itself both in vivo and in vitro (*2, 3*).

The rodlike structure of common TMV is constructed from 2130 chemically identical subunits which polymerize in very precise ways. According to Caspar, there are a number of stable aggregate states that the subunits may assume, all of which are in equilibrium with each other (*4*). The concentration of each of the stable aggregates depends on the protein concentration, the temperature, ionic strength, and *p*H of the medium. Low temperature, low salt, and protein concentration favor the monomeric state, whereas the reverse conditions favor polymerization (*5*).

Immune reactions studied in Ouchterlony plates revealed that a number of the aggregates could be recognized as distinct antigenic species (*6*). If the temperature of the TMV protein solution was raised from 0° to 30°C while all other conditions remained constant, a general progression of protein polymers was produced. These results suggested that polymerization was sufficient to introduce conformational changes in the antigenic site on the subunits. We report such antigenic changes accompanying the aggregation of TMV and PM2 protein.

Tobacco mosaic virus protein was prepared by treatment with cold acetic acid according to the method of Fraenkel-Conrat (*7*). The PM2 protein was extracted and purified by the method of Siegel *et al.* (*1*). Virus proteins at a final concentration of 5.0 mg/ml were emulsified with Freund's adjuvant. Rabbits were injected with 0.5 ml subcutaneously once a week for 3 weeks. The animals were rested for 1 month and then were immunized again by one subcutaneous and one intramuscular injection. Blood was obtained by cardiac puncture 10 and 11 days after the last injection.

The virus proteins were prepared at 1 mg/ml in 0.007*M* phosphate buffer at *p*H 7.0. Purified agar (Difco) at 1 percent was also prepared in 0.007*M* phosphate buffer. The antigens and antiserums were placed in the appropriate wells and incubated at different temperatures. Although clearly defined precipitin bands developed within 24 hours, the reactions were permitted to continue for 48 hours before the final reading was made. Representative data are given in Fig. 1.

At 4°C, broad precipitin bands appear close to the antiserum wells, suggesting a rapid diffusion of small antigen complexes in accord with the expected size of the polymers at low temperature (*4, 5*). The continuity of the major precipitin band between TMV protein and PM2 suggests that the antigen (or antigens) in question is identical in both cases.

A line can be seen between the TMV-protein well and the antiserum wells; this is not observed between PM2 and the antiserums. This precipitin line corresponds to an antigenic component absent in PM2. Although the antigenic component is missing from PM2, antiserum to PM2 precipitates that antigen as readily as antiserum to TMV does.

As the temperature is raised to 37°C, the putative small antigen in TMV protein disappears, although some of this small antigen remains in the PM2 well. Of greater significance, however, is the occurrence, at this temperature, of a narrow precipitin line in PM2 which is indistinguishable from its counterpart in TMV protein. The two antigens now appear very similar. At 42°C, the precipitation patterns are essentially identical.

Somewhat greater resolution of the aggregates and their attendant antigenic properties was obtained after immunoelectrophoresis in agar. The agar was prepared by melting 1 g of purified agar in a mixture of 25 ml of barbital buffer (*p*H 8.6, 0.1 μ) and 75 ml of H_2O. For electrophoresis we used an LKB 6800A instrument. Migration was conducted for 2 hours at 4 to 5 volt/cm at 4°, 25°, or 37°C. The apparatus, buffer, and all reagents were equilibrated at each temperature before electrophoresis. After each run, antiserum was added, and the plates were incubated for 48 hours at their respective temperatures. In one instance, the antigens were kept at 50°C for 2 hours; after this, electrophoresis was conducted at 37°C. The actual temperature of the migrating protein solutions in the agar in any of the above experiments was not determined. The series of temperatures merely suggests the range over which the antigens were exposed. Although no precise temperature values can be assigned, it is nevertheless clear (Fig. 2) that increasing the temperature from 4° to 50°C caused profound changes in the complex antigenic prop-

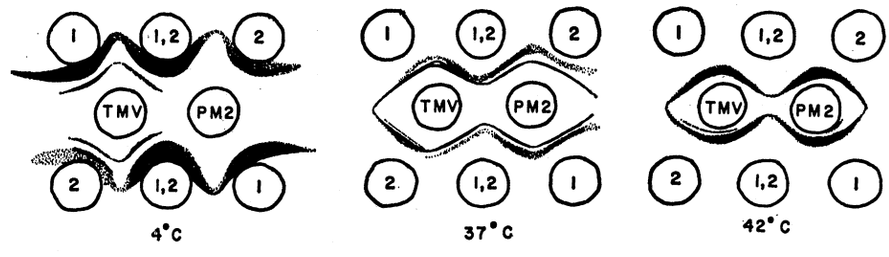

Fig. 1. Ouchterlony analysis of PM2 and TMV protein with their respective antiserums at different temperatures. Outer wells contain rabbit antisera. Antiserum to TMV, 1; antiserum to PM2, 2. Wells 1,2 contain artificial mixtures of equal volumes of the antiserums. Inner wells contain the viral antigens.

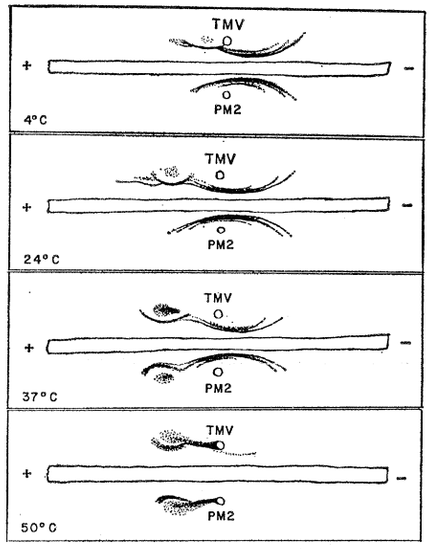

Fig. 2. Immunoelectrophoretic analysis of PM2 and TMV protein conducted at different temperatures. Antiserum to TMV protein was used to develop the precipitation patterns.

erties of both the wild-type TMV protein and the mutant PM2 protein. Below 37°C, TMV protein and PM2 are easily distinguishable. Apparently, a number of aggregates with greater electrophoretic mobility are present in the wild type at the lower temperatures. No counterpart to these antigens is seen in PM2. Raising the temperature to 37°C or above has the striking effect of making the two antigen preparations appear alike. This is in agreement with the results obtained by Ouchterlony analysis.

The defect associated with PM2 resides in the replacement of threonine by isoleucine in position 28 and in the replacement of glutamic acid by aspartic acid in position 95 of the polypeptide chain (*8*). Whether only one or both amino acid replacements are necessary for the expression of defectiveness is unknown. However, these data cast additional light on the consequences of the mutational event. The two amino acid replacements in PM2 protein are probably instrumental in preventing the subunits from polymerizing to the same degree as wild-type TMV protein at temperatures below 37°C. At 37°C and above, however, polymerization of PM2 appears normal by immunological criteria; this suggests that at least the conformation of the polypeptide at the antigenic surface of both TMV and PM2 is now the same. It is important to determine whether the defect in PM2 can be repaired by elevating the temperature so that the protein would polymerize with RNA and would aggregate into rodlike structures. Preliminary electron micrographs of PM2 protein incubated at 45°C indicate formation of somewhat more compact structures than the loose coils reported earlier (*2*).

IRVING RAPPAPORT
Department of Microbiology, New York Medical College, New York
MILTON ZAITLIN
Department of Agricultural Biochemistry, University of Arizona, Tucson

References and Notes

1. A. Siegel, M. Zaitlin, O. P. Sehgal, *Proc. Nat. Acad. Sci. U.S.* **48**, 1845 (1962).
2. M. Zaitlin and W. R. Ferris, *Science* **143**, 1451 (1964).
3. A. Siegel, G. J. Hills, R. Markham, *J. Mol. Biol.* **19**, 140 (1966).
4. D. L. D. Caspar, *Advances Protein Chem.* **18**, 37 (1963).
5. M. A. Lauffer, A. T. Ansevin, T. E. Cartwright, C. C. Brinton, Jr., *Nature* **181**, 1338 (1958).
6. I. Rappaport, A. Siegel, R. Haselkorn. *Virology* **25**, 325 (1965).
7. H. Fraenkel-Conrat, *ibid.* **4**, 1 (1957).
8. M. Zaitlin and W. F. McCaughey, *ibid.* **26**, 500 (1965); H. C. Wittmann, *Z. Vererb.* **97**, 297 (1965).
9. Supported in part by AEC contracts [AT-(30-1)-3557 and AT(11-1)-873] and grants from the American Cancer Society and NSF.
15 May 1967

Stretch Receptors in the Foregut of the Blowfly

Abstract. *Two bipolar neurons are located in a nerve branch connecting the recurrent nerve and foregut of the blowfly* Phormia regina. *Spike activity accompanying peristalsis or controlled enlargement of the foregut region is recorded from two cells in the recurrent nerve. The spikes are abolished by section of the nerve branch connecting recurrent nerve and foregut. It is concluded that the two neurons are the foregut stretch receptors predicted from results of behavioral experiments and vital to the regulation of feeding by the fly.*

A homeostatic mechanism controls the feeding behavior of the blowfly *Phormia regina* such that, in the presence of excess food, a constant amount is ingested per day (*1*). The essential element of the homeostatic mechanism is a negative feedback relationship whereby a consequence of feeding, filling the gut, reduces the probability of further feeding (*2*). Filling the gut activates receptors in the gut and body wall; the activity of these receptors inhibits in the brain the input from the external chemoreceptors which elicits feeding (*2, 3*).

The receptors in the gut wall are located in the region of the foregut (*2, 3*). Filling the other two gut segments, the hindgut and midgut, by enema has no effect on behavioral sensitivity to gustatory stimuli (*2*). Behavioral data indicate that the foregut receptors are stretch receptors (*4*). The foregut receptors have now been localized anatomically, and some of their electrophysiological characteristics have been determined.

The foregut-recurrent nerve complex in *Phormia* was dissected out; in some blowflies it was previously stained with leuco-methylene blue by the method of Zacharuk (*5*). Other preparations of freshly isolated recurrent nerve were stained with Giemsa stain for 15 minutes. These were examined immediately after isolation and vital staining. Suction electrodes were used for electrophysiological recording. The electrode assembly consisted of a micropipette coupled directly to a 1-ml syringe with a Tuohy adapter. A silver wire was inserted into the pipette to its tip and brought to the outside through the rubber gasket of the Tuohy adapter. The orifice of the electrode was adjusted so that a loop of nerve formed a snug fit when drawn into the electrode. Recording was differential between the electrode and the surrounding saline and was capacitatively coupled.

Controlled enlargement of the gut lumen was accomplished with an electrically driven microsyringe. A micropipette was converted into a microsyringe by filling it with fluid and inserting a close-fitting insect pin into its shank to act as a plunger. The insect pin was coupled to the cone of a loudspeaker so that short electrical pulses to the speaker were converted to short pulses of fluid from the tip of the micropipette. A gut segment was ligatured over the tip of the micropipette and was stretched by the hydraulic pulses.

A fine nerve branch arises from the ventral surface of the recurrent nerve and connects with the anterior region of the foregut. If this branch is cut at its termination on the foregut and is vitally stained with Giemsa stain for 15 minutes, two bipolar neurons can be seen within it (Fig. 1). These cells are typical insect neurons