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A Differential Ability of Strains of Tobacco Mosaic Virus to Bind Host-Cell Nucleoprotein[1]

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ONE OF THE PROBLEMS facing workers engaged in the purification of viruses and other proteins is the tendency for these large molecules to associate with other substances derived from the host tissue. It is recognized that a virus can form complexes with a wide range of substances (*1, 2*). Some of these complexes are rather stable whereas others are easily dissociable. Several examples of complexes between plant or animal viruses and host cell constituents have been cited by Pirie (*2*), who pointed out that such combinations are of limited interest unless they can be shown to be specific or related to some biological process or property. Tobacco mosaic virus (TMV) is particularly notorious in that different methods of purification yield preparations of varying degrees of color.

It is the purpose of this paper to report a selective ability of certain strains of TMV to form a stable complex with a nucleoprotein derived from the host cells, and to describe the conditions under which the complex may be dissociated.

It has been found possible to assign the strains of TMV being investigated in this laboratory into groups on the basis of biological, physical-chemical, and serological methods (*3*). Viruses of Group I are differentiated from each other by the symptoms they cause in *Nicotiana tabacum* and serologically, but the strains are indistinguishable with respect to the following characteristics: electrophoretic mobility, isoelectric point, ultraviolet absorption spectrum, and rate of inactivation by ultraviolet radiation. The strains within Group II are distinguishable also by symptoms and serology and are indistinguishable from each other by the above criteria, but the characteristics of the latter strains are sharply differentiated from the Group I viruses.

It is noteworthy that strains within Group I, of which common TMV is an example, are uniformly obtained as clear amber pellets on purification by ultracentrifugation in cacodylate buffer, whereas strains of Group II are colorless after the same treatment. The color remains associated with Group I viruses even after they are precipitated with acids or ammonium sulfate, or dialyzed at length against monovalent buffer salts in the range of pH 5–8 (e.g., acetate, cacodylate, and Veronal). The virus-color complex withstands freezing and thawing, and also precipitates as a complex when reacted with homologous antiserum. It cannot be dissociated by electrophoresis or by repeated ultracentrifugation. By these criteria the colored substance appears to be rather firmly bound to the virus molecule.

The virus-color complex can, however, be dissociated by exposure to various di- and polyvalent anions. The phenomenon was first noted in the course of attempts to remove the color enzymically. It was observed that virus in the control tubes, which was suspended in phosphate buffer during incubation, was obtained in nearly colorless pellets on centrifugation. Subsequent experiments showed that although the colored material after removal from the virus by phosphate treatment is readily separated by centrifugation, it cannot be separated by dialysis. Phosphate has been used in most of our investigations, although the effect is not limited to this ion.

After preliminary experiments indicated that the decolorization is not instantaneous at room temperature, the effect of temperature on the rate of the dissociation was studied. Clear amber pellets of strain

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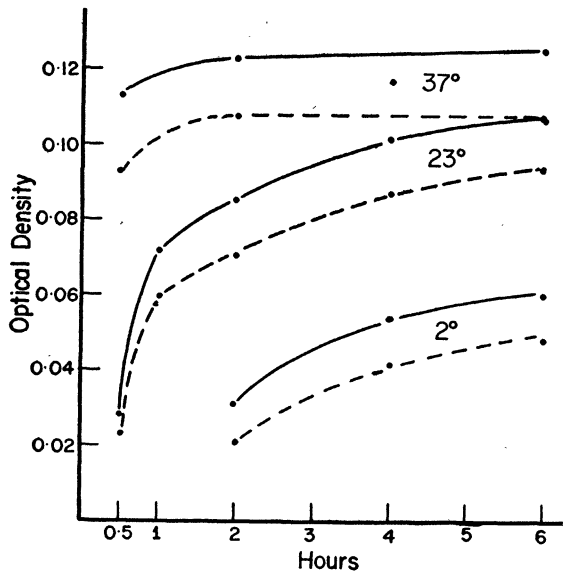

FIG. 1. The effect of temperature on the rate of decolorization of common TMV in 0.1 *M* phosphate buffer at pH 7.0. Optical density of supernatant liquid measured after removal of virus by ultracentrifugation. Solid line determined at 260 millimicrons, broken line at 280.

U1 (common TMV, a Group I strain), purified by four or more cycles of high- and low-speed centrifugation in cacodylate buffer, were dissolved in distilled water and the final protein concentration was adjusted to 0.25 percent. Forty-milliliter aliquots of this solution were centrifuged at 30,000 rpm in the No. 30 rotor of the Spinco preparative centrifuge for one hour and the supernatant solutions discarded. The pellets were rapidly dissolved by stirring in 40 ml of pH 7 phosphate buffer (0.1 *M*), and the virus solutions were held at 2°, 23°, and 37°. Ten-milliliter aliquots were taken after various time intervals, the reaction arrested by rapid cooling to 0°, and virus protein removed by centrifuging in a precooled No. 40 Spinco rotor at 40,000 rpm for one hour. The amount of colored material released from the virus was estimated by measuring the optical density of the supernatant solutions at 260 and 280 millimicrons. These wavelengths were selected somewhat arbitrarily because the color exhibits no absorption peaks in the visible or near ultraviolet except for a broad maximum near 260 millimicrons. Absorption in the ultraviolet is much greater than in the visible and thus gives a more sensitive measure of dissociation. The spectrophotometer blank was prepared from a virus sample dissolved in 10 milliliters of the same buffer and centrifuged in similar fashion at zero time. Thus, the observed optical density cannot be attributed to stray virus in the supernatant solution. The results, showing that rate of decolorization is markedly dependent on temperature, are presented in Fig. 1.

The effect of phosphate concentration on the rate of decolorization of virus is shown in Fig. 2. This experiment was conducted in exactly the same manner as the preceding one except that the temperature was held at 23° and the concentration of pH 7 phosphate buffer was varied as indicated. The dissociation is markedly dependent on phosphate concentration. It is of particular interest that the rate at 0.1 *M* is about ten times the rate at 0.05 *M*, since buffers in this concentration range are frequently used for virus purification. It is probable that all these samples would have been eventually completely decolorized, since exposure of the colored virus for a month to phosphate concentrations as low as 0.02 *M* at 4° has given colorless virus. Conversely, virus pellets have been obtained with no appreciable decolorization if worked up rapidly at low temperature in *M*/15 phosphate. It will be seen that wide variations in color may result from differences in technique of preparation, especially in the widely used 0.1 *M* phosphate buffer.

It was subsequently found that certain divalent and polyvalent anions have an effect equal to or greater than that of phosphate in causing the release of color. The ions were tested at pH 7 in 0.1 *M* solutions. The order of effectiveness of these compounds is: versene, citrate, and oxalate > phosphate, arsenate, malate, and tartrate > succinate, maleate, phthalate, sulfate, acetate, and malonate. Fumarate and cacodylate cause no detectable decolorization. It is of interest that (omitting the nonchelating inorganic ions and monovalent organic ions) the above order is the same as that for the stability constants of the calcium chelate complexes of these ions (*4*). It would seem likely that replacement of the colored material on the virus surface by the anions may be involved in the dissociation.

Removal of the colored material does not measurably change any of the physical-chemical character-

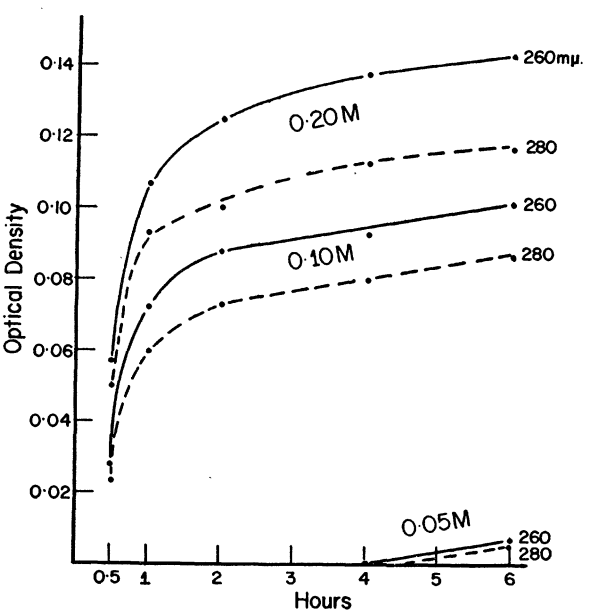

FIG. 2. The effect of phosphate buffer concentration on the rate of decolorization of common TMV at pH 7.0, temperature 23° C.

istics used to differentiate between the groups of strains. The infectivity of the virus is also unaltered after decolorization.

Colored material for analysis was obtained in the following manner. Several grams of the colored virus were prepared from infected leaves of *N. tabacum* by five cycles of alternate high- and low-speed centrifugation in cacodylate buffer and two additional cycles in double distilled water. At the end of this treatment, the supernatant solutions were water clear and the compacted virus pellets were dark amber in color. The pellets were then dissolved in 0.1 *M* phosphate at pH 7. The solutions were transferred to cellophane bags and dialyzed against a large volume of the same buffer at 4° for four days. As reported above, the dissociated colored material will not diffuse through the membrane, but can be separated from the virus by centrifugation. Contents of the dialysis bags were centrifuged at 30,000 rpm in the No. 30 Spinco rotor. The supernatant solution, containing the colored material in 0.1 *M* phosphate, was dialyzed against distilled water and then concentrated to a small volume at 4° by directing an air stream against the dialysis bag. The concentrated solution was centrifuged for three hours at 30,000 rpm to remove any final traces of virus and virus fragments, and then dialyzed against three changes of pH 6.83 cacodylate buffer ($\mu = 0.1$; NaCl 0.08 *M*, cacodylate 0.02 *M*) for six days.

The electrophoretic mobility in this buffer was -12×10^{-5} cm^2/v/sec compared with -8.9×10^{-5} for the parent virus. The boundaries migrated as single peaks with a trace of a minor component on either shoulder of the main peaks. The nitrogen/phosphorus ratio was determined to be 4.6 and 4.8 for two separate preparations of the brown material. Nitrogen was determined by the Nessler method and phosphorus by the Berenblum and Chain method (*5*). This ratio suggests a nucleic acid content of 34 percent, assuming the remainder to be protein with a nitrogen content of 16 percent. Pentose was estimated by the Meijbaum orcinol method (*6*), using yeast nucleic acid as a standard (*7*). The nitrogen/pentose ratio of about 0.9 further indicates a nucleic acid content of 37 percent. The colored material thus appears to be about six times as rich in nucleic acid as the parent virus which contains 6 percent nucleic acid. About 1/500 of the nitrogen of the virus was recovered in the brown material.

A sample of the colored material was treated with trichloroacetic acid, and the precipitate was hydrolyzed in a sealed tube at 120° for 16 hours with 6 *N* HCl. A two-dimensional chromatogram was made of an aliquot estimated (on the assumption that the colored material is 60 to 70 percent protein) to contain about 160 micrograms of amino acids. A parallel chromatogram was run on 160 micrograms of a mixture of equal parts of seventeen amino acids, and good separation was obtained. Most of the spots on the two sheets were superposable, and the approximate agreement in size and color intensity on spraying with ninhydrin indicated the presence of protein in the experimental sample at roughly the level proposed above. Thus, the material dissociated from the virus appears to be nucleoprotein in nature. Whereas the nucleoprotein may be intrinsically colored, it is perhaps more likely that its color is due to adherent small molecules. Whatever its nature, the coloring material appears to be firmly bound to the nucleoprotein, and no means for its removal have been found.

The colored nucleoprotein, once released, is not readsorbed by the parent virus. However, when the decolorized virus is mixed with a homogenate of uninfected fresh tobacco leaves and is then recovered in cacodylate buffer, the virus is again obtained as a characteristic brown pellet. This suggests that unaltered nucleoprotein is required and that the colored material may be irreversibly altered in the process of dissociation or subsequent handling, losing the necessary configuration for attachment to the virus. Under the same conditions strain U2 (a Group II strain) does not pick up any color from the fresh leaf homogenate. The observations suggest that the association of color with Group I viruses is due to configurational differences between the two groups of strains.

The problem of whether the colored virus complexes are artifacts of preparation or the colored material is normally associated with the virus in the host cell is difficult to resolve. It is clear at any rate that a marked difference in affinity for the colored material exists between the groups of strains that were originally differentiated by other criteria. Subtle differences in surface configuration of the virus molecules may account for these different affinities, just as they are assumed to account for immunological differences. It seems possible that selective affinities for other cellular components may exist and that these differences might be related to specific symptoms exhibited by virus infected plants.

The results of the present investigation seem of interest in that they demonstrate a differential ability of virus strains to bind a nucleoprotein derived from the host cell, they indicate the ability of di- and polyvalent anions to cause dissociation in a manner suggestive of displacement, and they may reconcile differences in degrees of coloration of tobacco mosaic virus preparations obtained by various workers using different methods of purification.

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