The Proteins Synthesized in Tissue Infected with Tobacco Mosaic Virus[1]

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WHEN tobacco mosaic virus (TMV) enters a suitable host cell, a new path of protein synthesis becomes established. Since one product of this pathway is a nucleoprotein identical with the inoculum, this event leads to reduplication of the virus. Biochemical characterization of the new metabolic pattern is an essential step toward understanding the mechanism of virus reduplication.

Recent investigations of this problem have shown that the initial source of nitrogen for TMV synthesis is the free ammonia present in the host tissue, and that nitrogen finally appearing in virus protein is first incorporated into an insoluble cell protein (*1*). These observations link the initial stages of TMV synthesis to the final product, but do not describe the intervening steps. The present paper summarizes results which serve to fill this gap in part. Reported are investigations which show that TMV biosynthesis is accompanied by the formation *de novo* of three other specific proteins and that the latter, while biologically inactive, are close immunochemical relatives of TMV. Analysis of the relationship between TMV synthesis and the synthesis of the nonvirus proteins outlines some of the steps which may be involved in TMV reduplication.

Since description of the products is often a useful way to characterize the productive apparatus, an effort was made to determine the number and nature of the new proteins synthesized in infected tissue as a result of inoculation with TMV.

The total soluble (i.e., in pH 7.0 phosphate buffer) protein complement of infected tobacco leaf was exhaustively fractionated and compared with the proteins found in otherwise identical uninfected leaf. Figure 1 shows the electrophoretic patterns obtained from various protein fractions when the total complement of soluble proteins is separated according to the scheme given. Four soluble proteins are found to be uniquely associated with TMV infection. One of these is the virus itself. As shown in the upper pattern of Fig. 1, the virus protein is readily isolated from the ultracentrifuge pellet obtained from the original extract. As expected, identical treatment of uninfected tissue yields no protein (see right hand uppermost pattern).

When the virus is removed from the original extract and the remaining low molecular weight proteins are brought to pH 3.4, a precipitate is obtained, which on further purification is found to contain only two electrophoretically distinguishable proteins. Identical treatment of uninfected tissue again gives no protein (center patterns Fig. 1). The slower of the components found in the infected preparation has an electrophoretic mobility averaging -3.4×10^{-5} cm^2/v/sec at pH 7.0 and is identical with component "B" previously reported by us (*2*) and with "protein X" of Takahashi and Ishii (*3*). This protein shall hereafter be designated B3. The second component (designated B6) has an average mobility of about -6.2 at pH 7.0, and is hitherto unreported.

When the low molecular weight proteins soluble at pH 3.4 are purified and examined electrophoretically, the results shown in the lowermost patterns of Fig. 1 are obtained. Infected tissue yields a single component with a mobility of about -4.7 at pH 7.0, whereas no protein is found in the comparable preparation from uninfected leaf. This component, hitherto unreported, has been given the designation A4.

Systemically infected tobacco leaf contains about 50–100 micrograms of B3 and B6, and about one-fourth that amount of A4 per gram wet weight. (The comparable value for TMV is about 2500 micrograms per gram.) The three nonvirus proteins have been found in all preparations made from infected leaf which has been permitted to attain a maximal level of TMV, and they have never been detected in normal leaf. Thus, like the virus itself these components appear in the plant as a specific consequence of infection with TMV.

To provide some insight into the origin of these proteins it is useful to compare their physical, chemical, and biological properties with those of the virus nucleoprotein. Table 1 reveals several significant relationships among these proteins. Unlike TMV, components B3, B6, and A4 are proteins of low molecular weight; their sedimentation constants are similar ($S=3$) and very small as compared with TMV ($S=ca.$ 100). Unlike TMV, none of the three proteins contains nucleic acid, as judged by their ultraviolet absorption maxima, and by the usual nucleic acid tests. Unlike the virus, the accessory proteins, when inoculated on TMV-susceptible plants (*N. tabacum* and *N. glutinosa*) are not infectious.

Despite these differences, the nonvirus proteins do show a kinship to TMV. When purified preparations of each of these proteins in phosphate buffer are

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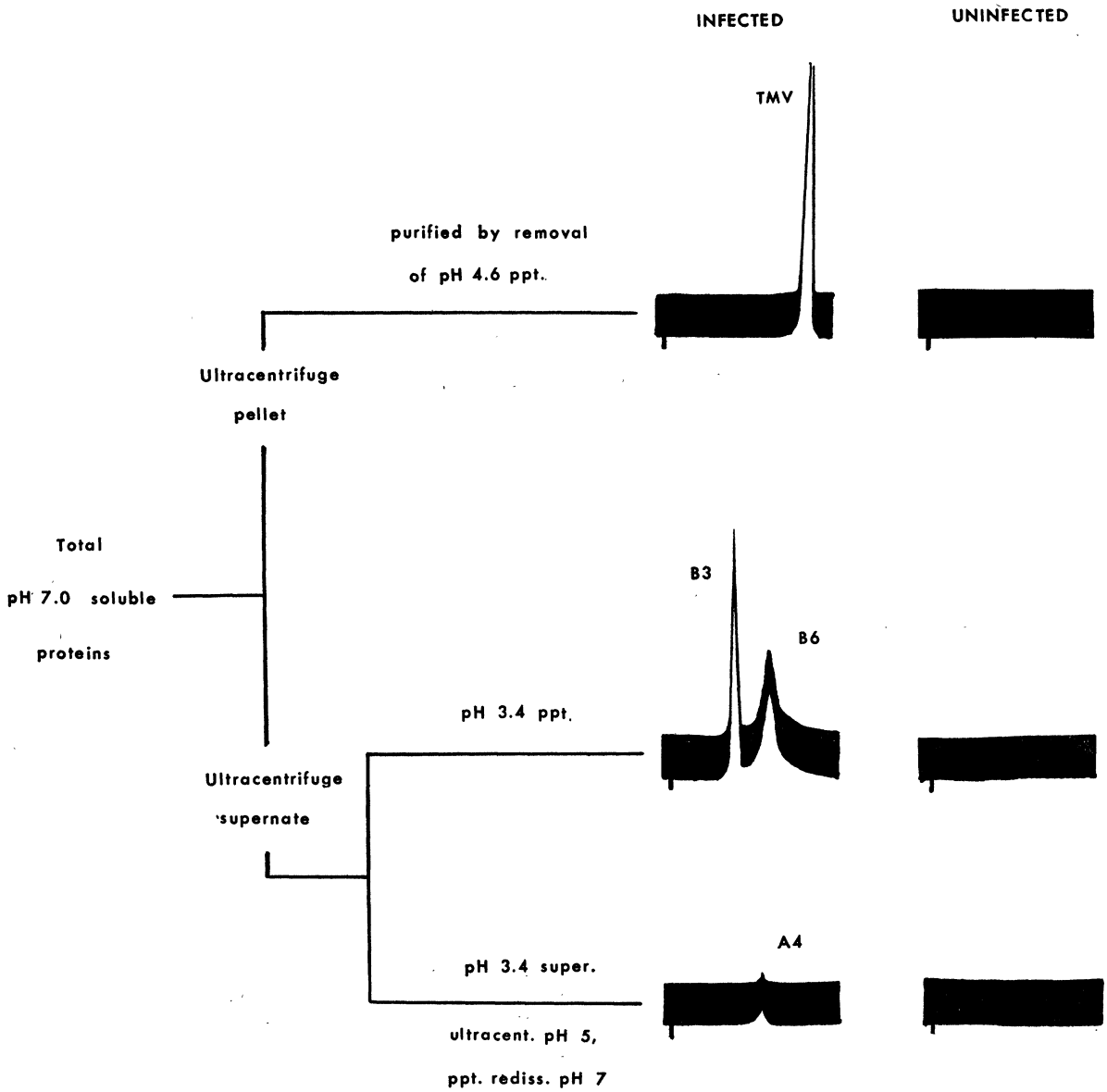

FIG. 1. Ascending electrophoretic patterns obtained from comparable fractions of soluble protein extracts of uninfected (right column) and systemically infected (left column) leaves of *N. tabacum* (variety White Burley). Movement is to the right. All preparations were in 0.05 *M* phosphate buffer, pH 7.0. Electrophoresis was carried out for 90 min at an average current of 8.5 ma. Ultracentrifugation was carried out for 1 hr at 104,500 × *g*.

brought to pH 5.0, they form high molecular weight aggregates with sedimentation constants in the range 100–200, which readily sediment in the ultracentrifuge. The polymerization of B6 and A4 is reversible, and the original low molecular weight proteins are easily regained when the preparations are returned to pH 7.0. However, the polymerization of protein B3 is irreversible under these conditions. This process appears to involve molecular rearrangement more deep-seated than mere physical aggregation, for the polymerized form has an electrophoretic mobility at pH 7.0 (−8.0) which is considerably different from that of the original B3 (−3.4). The polymerized form of B3 has been designated B8.

There is a suggestive similarity between the geometry of protein B8 and TMV. As can be seen from the electron micrographs (Fig. 2), both are rods of similar size and axial ratio. This observation agrees with the results of Takahashi and Ishii with polymerized "protein X" (*4*) and is further evidence of the identity of the latter with protein B3. Unfortunately, due to the instability of the polymerized forms of proteins B6 and A4, electron micrographs of these components are not yet at hand.

Thus, while the accessory proteins, as they occur in tissue extracts, are low molecular weight proteins, they are readily converted, *in vitro*, into macromolecules in the size range characteristic of TMV and

TABLE 1

PROPERTIES OF PROTEINS SYNTHESIZED IN THE LEAVES OF *N. tabacum* AS A RESULT OF INFECTION WITH TMV

Protein	Average electrophoretic mobility at pH 7.0 $cm^2/v/sec \times 10^{-5}$	Ultraviolet absorption maximum, mμ	Sedimentation constant, pH 7.0	Infectivity
TMV	−9.7	260	*ca.* 100	Infectious
A4	−4.7	280	3	None
B3	−3.4	280	3	None
B6	−6.2	280	3	None
B8*	−8.0	280	*ca.* 160	None

* B8 does not occur in the leaf as such, but is formed *in vitro* by polymerization of B3.

other plant viruses. These facts, especially the physical resemblance between protein B8 and TMV, might be taken to indicate that such aggregation actually occurs in the infected leaf, and that one or more of the low molecular weight accessory proteins serve as precursors of TMV. However, the aggregation, *in vitro,* of small globular proteins into a macromolecular fibrous product is by no means uncommon among proteins obtained from a variety of sources. For this reason, the conclusion that B3 or another of the accessory proteins is in fact a precursor of TMV must be supported by evidence more specific than that offered by a comparison of electron micrographs.

Data which more firmly establish close relationships among TMV and the nonvirus proteins have been obtained from immunochemical studies. A serum against TMV was prepared by injecting rabbits with an adjuvant emulsion of highly purified electrophoretically homogeneous TMV. The reaction of this serum with TMV, as determined by the quantitative precipitin technique of Heidelberger and Kendall (*5*) is shown in the upper curve of Fig. 3A. The curve is typical of the reaction of an antigen with its homologous serum, showing an equivalence zone at about 2 mg of antigen. Figure 3A (lower curve) also shows that anti-TMV serum will cross-react with protein B8. The reciprocal relationship is shown in Fig. 3B, which indicates that both B8 and TMV react with a serum prepared against an electrophoretically homogeneous preparation of protein B8.[2] Similar studies show that proteins B6, B3, and A4 cross-react with anti-TMV serum, and that protein B6 cross-reacts with anti-B8 serum (Fig. 4). Precipitin tests with comparable fractions from uninfected leaf show no reactivity with anti-TMV or anti-B8 serum (Fig. 4). Jeener and Lemoine have recently detected a nonvirus nucleic acid-free antigen which cross-reacts with anti-TMV serum in extracts of infected tobacco plants (*6*). It is not clear from their data whether the fraction studied was B3, B6, or a mixture of both proteins.

It is well established that the occurrence of immunochemical cross reactions between proteins is a clear indication of structural similarity (*7*). The above results are evidence, therefore, of close structural relationships among TMV and proteins B3 (or its polymer B8), B6, and A4 which do not extend to any normal tobacco protein. Previous observations (*1*) have shown that the protein fraction from infected leaf that contains B3 and B6 rapidly incorporates N^{15} from isotopically labeled host ammonia. Like the virus, these proteins appear to be synthesized *de novo* from ammonia in infected tissue.

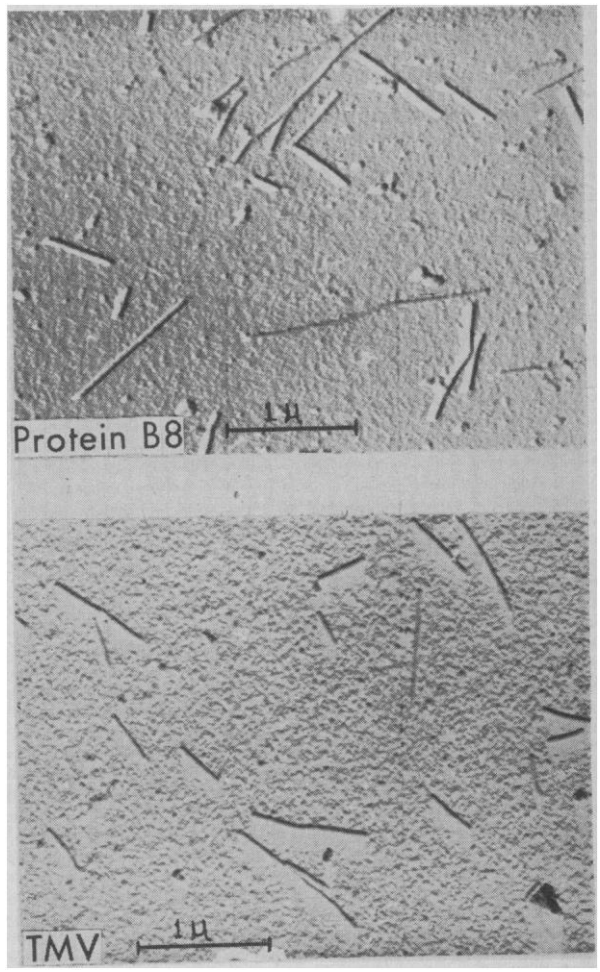

FIG. 2. Chromium-shadowed electron micrographs of TMV and protein B8 obtained from pH 7.0 solutions of these proteins.

It can be concluded therefore that in common with TMV, the nonvirus components that appear in the leaf as a result of infection are specific products of the new paths of protein synthesis brought into action by the entry of the virus inoculum into the host cell. Since the nonvirus proteins appear to be functional components of the process of virus reduplication, any mechanism proposed for the biosynthesis of TMV

[2] These cross reactions have been confirmed by tests conducted in agar diffusion tubes.

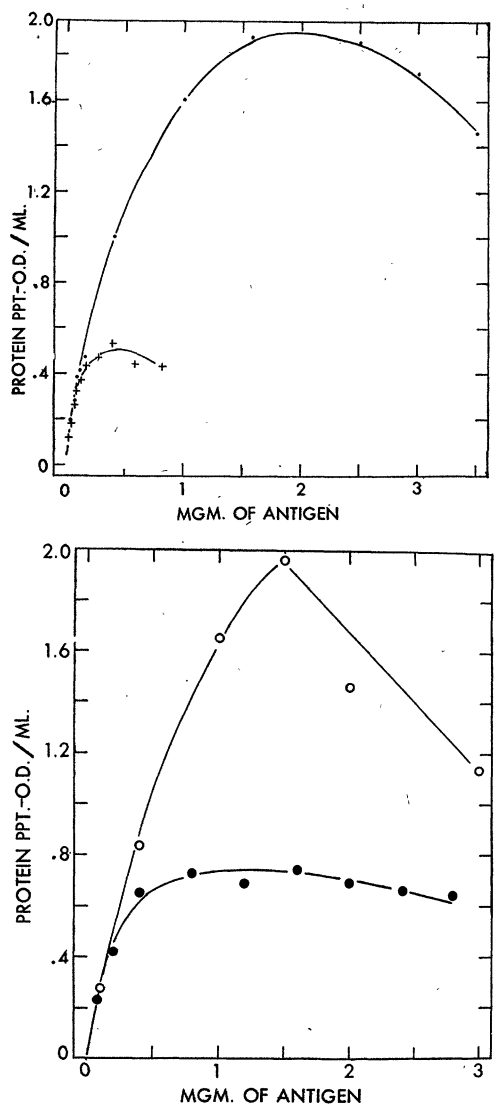

FIG. 3. Precipitin reactions of TMV and protein B8 with rabbit sera prepared against electrophoretically homogeneous TMV and B8. (Above) Precipitate formed by reacting 0.05 ml of anti-TMV serum with indicated amount of TMV (points, upper curve) and B8 (plus signs, lower curve). Optical densities are at 750 mμ and represent color due to Folin phenol reaction of precipitates in 1 ml of reagent. (Below) Precipitate formed by reacting 0.05 ml of anti-B8 serum with indicated amounts of TMV (open circles, upper curve) and B8 (closed circles, lower curve). Ordinate as for 3A.

must also account for the concomitant synthesis of these accessory proteins.

To describe the relationships between the synthesis of TMV and B3, B6, and A4 the amounts of these proteins present in infected tissue after various periods of time following inoculation have been determined with the aid of the immunochemical reactions just described. The results of one such experiment are shown in Figs. 4 and 5. In this experiment, a series of comparable uninfected and infected leaf blade samples were cultured in nutrient under constant conditions for 371 hr following the time of inoculation. Periodically, infected and uninfected tissue samples were removed, homogenized, and fractionated to yield isolated TMV, A4, B6, and B8 (by polymerization from B3). TMV was determined chemically. The A4 component was estimated by reacting an increasing amount of this fraction with anti-TMV serum and determining the precipitates formed. Similar reactions were carried out between aliquots of the B6 and B8 fractions and anti-B8 serum.

Typical results of such determinations are shown in Fig. 4 for tissue extracted at 371 hr after inoculation.

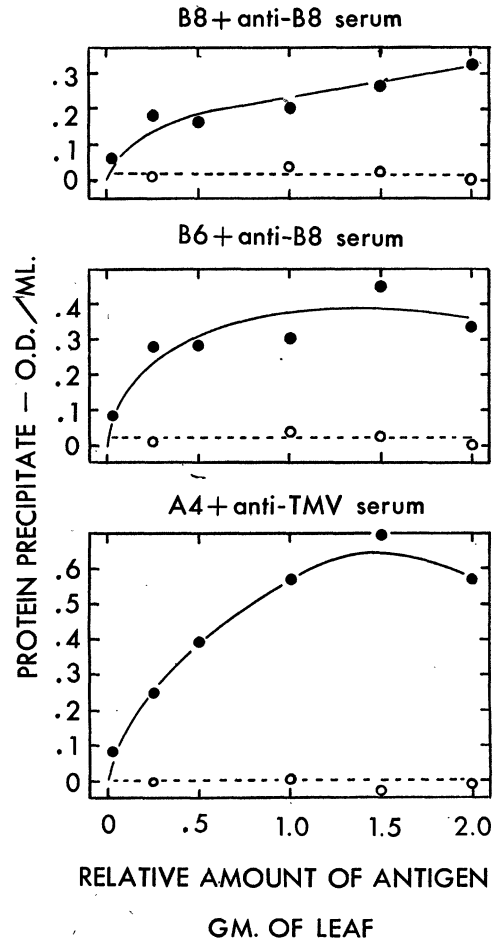

FIG. 4. Precipitin reactions between protein fractions from otherwise identical uninfected (open circles, broken lines) and infected (closed circles, solid lines) tobacco leaf tissue cultured for 371 hr after inoculation with TMV. Ordinate as for Fig. 3. Abscissa indicates the amount of tissue from which the given antigen sample was obtained.

Precipitin reactions were obtained from all three accessory protein fractions derived from infected tissue. On the other hand, no reactions occurred with the same fractions derived from the otherwise identical uninfected tissue. This is confirmatory evidence that the three nonvirus proteins are found in infected leaf but not in normal leaf.

From the initial slopes of curves such as those

shown in Fig. 4 it is possible to estimate the amount of each protein present in a given tissue sample. Measurements of this kind were made for each of the three fractions isolated from uninfected and infected tissue at various times after inoculation. At all times the fractions derived from uninfected tissue gave no precipitin reaction. The results obtained from infected tissue are shown in Fig. 5 together with a curve describing the TMV content of the tissue.

It is apparent that the rates of production of the nonvirus proteins are significantly related to the appearance of TMV itself. None of the nonvirus proteins is detectable until about 220 hr after inoculation, when the TMV content has already attained about one-third its maximum level. Protein A4 rises most rapidly in amount; more than half the final A4 content appears in the 24-hr period just preceding 220 hr. Protein B6 appears somewhat more slowly than A4 at first, but the rate of formation continues at its initial level even after the rate of appearance of both TMV and A4 have leveled off. Protein B3 (determined in the form B8) is not detectable until 280 hr after inoculation, and it is thus the last of the three proteins to occur in the tissue.

The most striking result of these observations is that the nonvirus proteins, A4 in particular, appear quite suddenly at a time which is well beyond the point when TMV is first detected. This suggests that the appearance of the nonvirus proteins has been brought about by some qualitative change in the character of the biochemical mechanisms already engaged in production of TMV; Fig. 5 indicates that this change occurs at about 200 hr after inoculation. The fact that one of the chief differences between TMV and the nonvirus proteins is the latters' lack of nucleic acid suggests that some change in nucleic acid metabolism at 200 hr may act as the stimulus for the abrupt emergence of new products in the infection process at this time.

This suggestion is supported by some preliminary observations on the nucleic acid metabolism of infected and uninfected leaf tissue which were made during the course of the experiment described in Fig. 5. It has been found that a nucleoprotein containing about 20–30 per cent pentose nucleic acid can be extracted from the buffer-insoluble protein of tobacco leaf by a strong salt solution. The amount of this nucleoprotein present in the uninfected and infected tissues just described was determined; the differences in the amounts present in comparable uninfected and infected tissue at various times after inoculation are plotted in the lower curve of Fig. 5. This curve shows that excess nucleoprotein develops in infected tissue before TMV appears (at about 100 hr). Then, as TMV begins to be formed, this excess is reduced until it falls to zero at 203 hr. It is just after this point that the nonvirus nucleic acid-free proteins begin to appear in the tissue.

Determinations of the differences in pentose nucleic acid content of the insoluble nucleoprotein fraction of the tissues discussed above gave results which paralleled those described in the lower curve of Fig. 5.

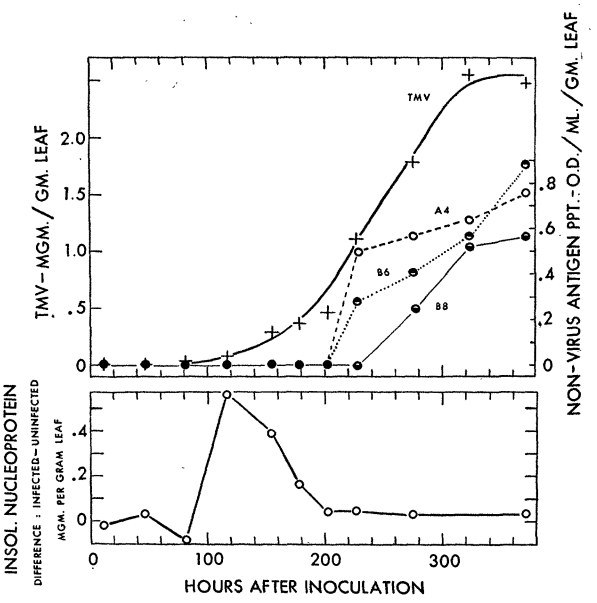

FIG. 5. The amounts of various proteins found in nutrient-cultured tobacco leaf tissue at various times after inoculation with TMV. Virus determined chemically. Proteins A4, B6, and B8 determined immunochemically as described in Fig. 4. The lower curve represents the differences between the nucleoprotein extracted by 10% NaCl solution from insoluble fraction of infected tissue and comparable uninfected samples. Values given are based on micro-Kjeldahl nitrogen determinations.

In summary, the foregoing results show that in addition to TMV, infected tissue synthesizes *de novo* three noninfectious nucleic acid-free proteins which, on the basis of immunochemical cross reactivity, are closely related to the virus. The nonvirus proteins, like TMV itself, therefore appear to be produced specifically as a result of the new path of protein synthesis induced in the host by inoculation with TMV. However, the accessory proteins are not formed in parallel with TMV, but appear after a rather sudden change (probably associated with depletion of a nucleic acid store) in the character of the biosynthetic process.

It can be concluded that each of the nonvirus proteins plays some specific part in the process of TMV reduplication. For each of the proteins three alternative propositions are consistent with the data:

Alternative 1. Nonvirus protein and TMV are sequential products of the same protein-synthesizing mechanism, i.e., nonvirus protein may act as an actual precursor of TMV. This proposition would mean that the nonvirus protein is produced at all times by the TMV-synthesizing mechanism and is converted to TMV at a variable rate. The early absence of the nonvirus protein might be accounted for by rapid and complete conversion to TMV. The sudden appearance at 200 hr might result from depletion of the previously synthesized nucleic acid required to convert it to TMV. The possible assignment of precursor roles to one or more of the nonvirus proteins introduces an interest-

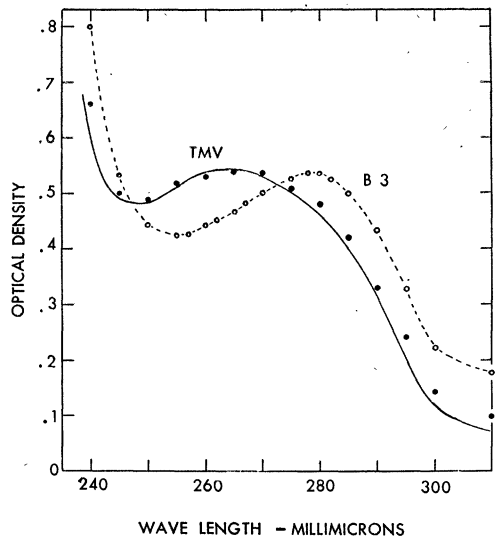

FIG. 6. The ultraviolet absorption spectra of protein B3 (open circles, dotted line), TMV (solid line), and a complex of protein B3 with nucleic acid prepared from TMV (closed circles).

ing experimental approach to the problem of TMV synthesis: an attempt to induce infectivity in the nucleic acid free presumed precursor by the addition, *in vitro*, of nucleic acid obtained from TMV. Proteins frequently form nonspecific *in vitro* complexes with nucleic acid. The absorption spectrum of such a complex between B3 and TMV nucleic acid is illustrated in Fig. 6. This product was not infectious. It is, of course, presumptuous to suppose that the mere mixing of the two components in simple solution would accomplish what must be in the living cell a highly specific, structurally oriented process. Nevertheless, such studies may shed light on the intracellular behavior of the nonvirus proteins.

Alternative 2. Nonvirus protein is an alternative rather than sequential product of the mechanism that produces TMV and appears during the course of virus synthesis as a result of a sudden loss in specificity of the synthesizing processes.

If this were the case, a rather close resemblance of the new products (i.e., A4, B6, B3) to the old one (TMV), such as that revealed by immunochemical cross reaction, is to be anticipated. Such an event might be usefully thought of as a mutation, and the nonvirus protein as a noninfectious "mutant" of TMV, or perhaps a low molecular weight breakdown product of such a "mutant."

Alternative 3. Nonvirus protein is part rather than product of the TMV-synthesizing apparatus of the cell. If this were the case, our observations would show that: (a) the parts of the apparatus represented by nonvirus protein are synthesized *de novo* subsequent to inoculation and do not antedate the entry of the inoculum; (b) these parts of the virus-synthesizing mechanism bear a close structural similarity to TMV; (c) the soluble nonvirus protein detected in tissue extracts is probably sloughed off from an insoluble cell component; and (d) this degeneration of the TMV-synthesizing apparatus begins at about 200 hr after inoculation, and is possibly associated with depletion of a source of pentose nucleic acid.

Experiments bearing on these alternative propositions are in progress.

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Birmingham Conference on Nuclear Physics

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AN international conference on nuclear physics, arranged by the departments of physics, electron physics, and mathematical physics, was held at the University of Birmingham, July 13th to 18th, 1953. It was attended by about two hundred fifty visitors, thirty of them from America. The chief topic of the conference was the complex nucleus; cosmic rays and meson physics proper were not discussed, nucleon-nucleon collisions only briefly. This wise concentration allowed a reasonably detailed treatment of the different items on the program. Each session was opened by a summarizing talk on the present status of the subject which was followed by short contributions and ample discussion.

Rather than reviewing the whole conference chronologically, some of the results of the discussions will be presented. References will be given sparingly, and will pertain only to the speaker.

Nuclear Radii. The $2p$-$1s$ transition of μ^--mesons, the diffraction pattern for high-energy particles, and the isotope shift seem to indicate nuclear radii somewhat smaller than the usual value of 1.45 to 1.50×10^{-13} $A^{1/3}$ cm. An irregular variation is found between Be^9 and O^{16}, both from neutron cross sections and the