LOCALIZATION OF DNA AND PROTEIN IN TIPULA IRIDESCENT VIRUS (TIV) BY ENZYMATIC DIGESTION AND ELECTRON MICROSCOPY

RICHARD S. THOMAS, Ph.D., and ROBLEY C. WILLIAMS, Ph.D.

From the Virus Laboratory, University of California, Berkeley. Dr. Thomas' present address is Western Regional Research Laboratory, United States Department of Agriculture, Albany, California

ABSTRACT

De-embedded ultrathin sections of ethanol-fixed Tipula Iridescent Virus particles were incubated with pepsin at pH 1.8, trypsin at pH 7.7, and DNase at pH 7.7. The outer shell of the particles, but not an inner core, was removed by the action of pepsin. Conversely, the inner core, but not the outer shell, was removed by the action of trypsin and DNase in combination, but not by either enzyme acting alone. These results are taken to mean that the outer shell of the particles is protein in nature and the inner core is nucleoprotein. Whole virus particles were also exposed to the same 3 enzymes. Trypsin and/or DNase had no effect on the whole particles, while pepsin at pH 1.8 digested away the outer shell of the particles and released an intact core, resistant to pepsin. The protein nature of the digested outer shells and the nucleoprotein nature of the released cores were confirmed by ultraviolet absorption spectra. Chemical analyses showed that the cores contain 89 per cent of the whole virus phosphorus but only 35 per cent of the nitrogen, while the outer shells contain only 5 per cent of the phosphorus but 63 per cent of the nitrogen. On the basis of nitrogen:phosphorus ratios the composition of the cores is estimated to be about 30 per cent DNA and 60 to 65 per cent protein.

INTRODUCTION

Tipula Iridescent Virus (TIV) is a natural pathogen for the larvae of Tipula paludosa, although it has been recently shown (1) that other dipterous species, and even other insect orders, can become infected through mechanical inoculation. The virus can be purified quite readily since it grows to high titre in its natural host and has a size sufficiently different from normal host material to allow it to be separated easily by centrifugation (2). The virus particles are uniform in size, having a diameter of 130 mµ when frozen-dried, and are distinctively icosahedral in shape (3) (see Fig. 1). They are the largest known virus particles to have such uniform morphology, having a particle weight of $1.2 \times 10^8$ (4). A microcrystalline array of TIV is beautifully iridescent, when illuminated with white light, owing to Bragg reflections which occur between crystal planes that are approximately $\frac{1}{2}$ wave length apart (5).

Chemical analysis of purified preparations of TIV (4) indicates that it contains 12.4 per cent DNA, 5.2 per cent lipid (mostly phospholipid), the remainder being protein and, perhaps, phosphoprotein. There appears to be no RNA nor polysaccharide in the virus.

Sections of osmium-fixed pellets of TIV, when examined in the electron microscope, show a nucleoid, or core, about 90 mµ in diameter. Surrounding this is an outer shell, or envelope, of slightly lower electron-scattering power (see Fig. 2). In sections of some preparations there appears an outer limiting membrane (6). On the
Explanation of Figures

All micrographs are at ×50,000 and show the same object, Tipula Iridescent Virus (TIV), when subjected to various treatments. With the exception of Fig. 2, all micrographs show uranium-shadowed specimens. The obliquity of shadowing is indicated by “tan−1”: the ratio of the height of an object to the length of its shadow.

FIGURE 1

Particles of frozen-dried TIV. The particles are icosahedra, as demonstrated by their contours and the shapes of their shadows.

FIGURE 2

Section of pellet fixed in osmium tetroxide. Some indications of a dense core can be seen in most of the centrally-transected particles.

whole, the appearance of TIV in sections is similar to that of many other large "spherical" viruses.

Since the internal composition of the virus is morphologically inhomogeneous (a shell and a core), and since it is large, uniform, and well characterized chemically, it provides an apt object for cytochemical analysis leading to the localization of the DNA, the protein, and the lipid. Such localization can be investigated by following the action of different enzymes on sections of the virus and on whole virus particles.

MATERIALS AND METHODS

Virus

The sample of TIV used in these experiments was the same as that used earlier for chemical analyses (4). It was a highly purified suspension containing about 10 mg TIV/ml in 0.003 M ammonium bicarbonate. The TIV at this concentration was quite turbid, a property which was useful in gauging the extent of enzymatic digestion of whole virus particles (see below).
Enzymes

Crystalline enzymes were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Relatively concentrated solutions were used in all cases to insure that the enzyme concentration would not be rate limiting. For digestion of virus particles within thin sections, pepsin (twice crystallized from ethanol, lot PM 539) was routinely made up to 0.5 mg/ml in 0.05 M HCl (7); trypsin (crystallized, lyophilized, salt-free lot TL 576) was dissolved 0.5 mg/ml in either 0.1 M sodium borate plus 0.0025 M calcium chloride at pH 7.6 (8), or 0.05 M sodium veronal plus 0.01 M magnesium chloride plus 0.0025 M calcium chloride at pH 7.7; DNAse (once crystallized, lot D678-9) was dissolved 0.5 mg/ml in either 0.1 M sodium acetate plus 0.01 M magnesium chloride at pH 6.4 (9), or in the same 0.05 M sodium veronal buffer used for the trypsin. The sodium veronal buffer was formulated specifically to be suitable for both enzymes.

For digestion of whole TIV particles in suspension the enzyme solutions were the same except that the buffer concentrations were doubled to allow 1:1 dilution with the virus suspension (see below).

Electron Microscopy

All electron micrographs were taken with an RCA model EMU-3 instrument equipped with a 35 μ objective aperture and operated at 50 kv.

Preparation of Sections

Sections of TIV sufficiently thin for electron microscopy were prepared in the following way for the experiments with enzyme digestion. A small aliquot of the stock TIV suspension was centrifuged 30 minutes at 8,000 g (Servall SS-1 centrifuge) in a microcentrifuge tube to pack the virus particles into a microcrystalline pellet (2), about 1 mm in diameter. The pellet was either fixed in situ by 1 per cent buffered osmium tetroxide for 1 hour, or the osmium fixation step was omitted and fixation was effected by initial contact with ethanol. Dehydration in situ was effected by successive 30-minute treatments with 50–75–95–100 per cent ethanol, and 100 per cent methacrylate monomer (25 per cent methyl, 75 per cent butyl). The pellet was centrifuged throughout the fixation and dehydration periods, with the orientation of the centrifuge tube and the speed of the rotor duplicating as nearly as possible the conditions of initial centrifugation. This procedure maintained the microcrystallinity within the pellet, a feature proving desirable when sections were examined. The dehydrated pellet, now quite rigid, was removed from the microcentrifuge tube and transferred to fresh methacrylate monomer (25 per cent methyl, 75 per cent butyl containing 2 per cent benzoyl peroxide) in a gelatin rectal capsule. Polymerization was carried out under N₂ at 50°C and sections were cut with a diamond knife on a Porter-Blum microtome. After the sections had been spread by exposure to xylene vapor (10) they ranged in interference color from grey to light gold. They were picked up on carbon-filmed 200-mesh copper grids. The methacrylate was removed by immersing the grids for 1 hour in benzene, and the sections were rehydrated by passing them briefly through a series of graded mixtures of benzene-ethanol, pure ethanol, and ethanol-water. They were finally passed through a dilute solution of bovine plasma albumen, rather than pure water, since the carbon films would not wet in the latter. A small droplet of liquid was allowed to adhere to the grids during each rehydration transfer so that the sections would at no time become dry. This precaution prevented any unnecessary distortion of the exposed transsections of the virus particles owing to forces of surface tension during drying.

Digestion of Sections

For digestion of the rehydrated sections, grids were transferred to the enzyme solution and incubated for 60 minutes at 35°C in a water bath. Other grids were incubated in the appropriate buffer as controls. They were then passed back through distilled water and the graded mixtures of water-ethanol and ethanol-benzene to pure benzene, and dried in air. The sections were dried out of benzene rather than water because of the lower surface tension of the former. The grids were then sprayed with polystyrene latex particles, followed by uranium shadowing, and placed in the electron microscope for examination.

Digestion of Whole Particles

To determine the effect of enzymes on the morphology of intact TIV particles, the stock virus suspension was mixed 1:1 with enzyme solution and incubated 30 minutes at 35°C in a
water bath. The resulting suspension was observed visually for decreases in turbidity and subsequently examined in the electron microscope. Control preparations consisted of the virus suspension after mixing with, and incubation in, the appropriate buffer. The electron microscopic examination of the enzymically treated virus particles was made either on sprayed and shadowed preparations or on sections of pellets of the particles.

For sprayed preparations of the particles it was necessary that the material be finally dried from a completely volatile medium to avoid the masking effects of dried salts. Thus, DNase- and trypsin-treated particles, and their controls, were pelleted (30 minutes at 8,000 g, Servall SS-1 centrifuge) and resuspended in 0.003 M ammonium bicarbonate. This procedure was not necessary for pepsin-treated particles, and their controls, since the original medium, 0.05 N HCl, is volatile. The particles were diluted 1:25 (with respect to the digest) with the appropriate volatile medium, sprayed with an atomizer onto Formvar-filmed grids, and air dried. The grids were then sprayed with polystyrene latex particles and shadowed with uranium.

For thin-section preparations, the enzymically treated particles and controls were pelleted, osmium-fixed, and otherwise handled as described above. The sections were examined, however, without removal of the embedding material.

Chemical Analysis

To investigate the chemical alterations brought about by enzymatic digestion of the particles, the complete digest, minus an aliquot set aside for analysis, was centrifuged 1 hour at 8,000 g (Servall SS-1 centrifuge) to pellet any large particles which it might contain. The supernatant was carefully withdrawn and saved, and the pellet was resuspended to the original volume in the appropriate enzymic suspending medium. The complete digest, supernatant, and resuspended pellet were analyzed for total nitrogen (11) and total phosphorus (12), and their ultraviolet absorption spectra were determined against appropriate blanks. For the latter measurements, the large particles in the whole digest and in the resuspended pellet were first dissolved in 1 per cent sodium dodecyl sulfate (4) to eliminate turbidity. All 3 preparations were diluted with distilled water to a degree appropriate for measuring optical density.

RESULTS

Appearance of Untreated Sections

The localization of the DNA and protein within TIV particles is revealed the most graphically by a comparison of the appearance of enzyme-treated sections with untreated sections. The standard untreated section was one obtained from a virus pellet which had been fixed only by alcohol dehydration and embedding, and from which the embedding material had been removed. Pellets fixed in the usual way with osmium tetroxide before dehydration yielded preparations which showed better preservation of morphology but they were completely resistant to enzyme digestion. Figs. 3 and 4 show de-embedded, shadowed sections of osmium-fixed and alcohol-fixed pellets, respectively. The osmium-fixed section, except for the absence of embedding methacrylate and for the presence of shadows, is similar to that shown in Fig. 2. It is easily seen that the sections in both cases are much thinner than the diameter of the virus particles. This apparent thinness may be due in part to flattening brought about by

Figure 3

Section of osmium-fixed pellet; methacrylate removed. In a number of the particle transections the peripheral envelope of the particle can be distinguished from the central core. A fold in the section allows some transections, doubled up, to be seen on edge. Tan $^{-1} \frac{1}{2}$.

Figure 4

Section of alcohol-fixed pellet; methacrylate removed. Preservation of structure is imperfect, but an artificial separation of the peripheral envelope from the central core allows the two structures to be more readily distinguished. Tan $^{-1} \frac{1}{4}$.
surface-tension forces during de-embedding. Some evidence for this notion is seen in the osmium-fixed preparation (Fig. 3) where the measured diameter of the largest particle transections (measured perpendicular to the direction of cutting) is about 150 m\(\mu\), or 15 per cent greater than the corresponding diameter of intact particles (130 m\(\mu\)). However, the thickness of some sections, estimated from interference color (13) before de-embedding, was probably no more than 20 m\(\mu\), or about one-seventh the diameter of the particles. The crystalline nature of the pellets is also readily apparent from the geometrical array of the particles. It will be noted, especially in Fig. 4, that the crystal planes in which the particles lie pass gradually in and out of register with the plane of the section; thus transections of individual particles are not random in the degree to which they are non-diametral, or eccentric, as is the case in sections of non-crystalline pellets, but rather their degree of eccentricity follows a progressive sequence along rows. This property of the sections is helpful, as we shall see, in interpreting the results of enzyme action.

In the osmium-fixed preparation (Fig. 3) hexagonal profiles which are compressed in the direction of cutting can occasionally be seen, as might be expected from a section of an icosahedron. Close inspection reveals what appears to be the counterpart of the electron-dense central cores seen in sections with embedding intact. These are outlined, in favorable instances, by a thin space where the outer shell of the particle has separated from the core, or by a depression on the surface of the particle transection.

In the alcohol-fixed preparation (Fig. 4) the outer shells of the particles seem to be somewhat frayed and swollen, and hexagonal profiles are less readily recognized. The cores, however, are more easily distinguished as a result of their greater separation from the outer shells. The cores of centrally transected particles seem to be about 100 m\(\mu\) in diameter. When this figure is corrected for the flattening artifact it becomes about 90 m\(\mu\), and agrees with the approximate diameter of the cores seen in embedded sections.

**Enzymatic Action on Transected Particles**

(a) Effect of Pepsin: The result of treating sections of ethanol-fixed material with pepsin in 0.05 N HCl is shown in Fig. 5. Pepsin appears to digest away the transected outer shells of the particles without attacking the cores. The round objects remaining in the sections are not bordered by annular rings and they are mostly about 100 m\(\mu\) in diameter, in agreement with the core diameter of untreated sections. Where the diameter is smaller it is clear from the particle array that the object is an eccentric transection. It is interesting to note that there is much empty area in the section. This apparently represents regions where the particle transections were so eccentric as to include only a portion of the outer shell of the particle which was subsequently digested completely away.

Treatment with 0.05 N HCl alone had no obvious effect on the sections, except perhaps to make the particle transections appear somewhat more frayed than they appear in Fig. 4.

(b) Effect of DNase: The result of treating sections with DNase was to leave the particle transections somewhat more frayed than they appear in Fig. 4, but with no greater alteration than was apparent in the buffer controls.

(c) Effect of Trypsin: A section treated with trypsin was shown in Fig. 6. In this case there is an effect, but it is somewhat inconsistently obtained and is certainly rather odd in character. Frequently, but not always, small mounds of material are seen precisely on the centers of the transected particles. The height and shape of the mounds can be judged by comparing transected particles which have mounds to adjacent ones

**Figure 5**

Section of alcohol-fixed pellet; methacrylate removed, followed by treatment with pepsin. Only the transected cores remain in the section. Tan\(^{-1}\) 3\(\frac{1}{2}\).

**Figure 6**

Section of alcohol-fixed pellet; methacrylate removed, followed by treatment with trypsin. Most of the transected particle structure remains, but some material (presumably the enzyme) has been deposited on many of the cores. Tan\(^{-1}\) 3\(\frac{1}{2}\).
which do not. This phenomenon is never seen in buffer controls, and hence it is likely that the mounds are adsorbed enzyme protein. For reasons unknown, the enzyme seems to have a special affinity for the transected cores of the particles. There appears to be no lytic action of the enzyme, however.

(d) Effect of Trypsin Plus DNase: Sections treated with trypsin plus DNase, a 1:1 mixture in veronal buffer, are shown in Figs. 7 and 8. In contrast to the action of either alone, the 2 enzymes acting together have a pronounced lytic effect and the result is quite striking. The core substance of the particle transections is digested away, leaving behind rings which apparently represent the outer shell material of the particles. The result thus seems to be the converse of that achieved with pepsin alone. The identification of the observed rings with the outer shells of the particles is secured by noting that the diameter of the largest rings (representing centrally transected particles) is about 150 m/z. The impressive uniformity of the enzyme action is best seen in thin sections, such as shown in Fig. 7. The small, round structures seen here and there undoubtedly represent only the tops (or bottoms) of particles included in the section, as can be seen from their positions in the array. The tops or bottoms should be more frequently included in thicker sections, and this is seen to be the case in Fig. 8 where the section thickness approaches the diameter of the particles.

Enzymatic Action on Whole Particles

(a) Action of Trypsin and/or DNase: Treatment of intact particles in suspension with trypsin, or DNase, or a 1:1 mixture of trypsin plus DNase had no apparent effect on their morphology. The turbidity of enzyme-treated preparations and the appearance of treated particles, either intact or sectioned, in the electron microscope were indistinguishable from incubated, or even unincubated, buffer controls. These experiments thus confirm the DNase- and trypsin-resistance of the particle shell when exposed to these enzymes in thin sections. That the central core was not attacked is quite understandable in view of the resistant outer coat which undoubtedly affords protection to the core.

(b) Effect of HCl and of Pepsin: In contrast to trypsin and DNase, pepsin had a pronounced effect on the particles, as did also the 0.05 N HCl medium alone. The turbidity of incubated suspensions in 0.05 N HCl was noticeably less, and in pepsin solution markedly less, than that of incubated controls in neutral buffer. The decrease in turbidity of both preparations could be seen after 5 minutes' incubation, and in both cases it appeared to reach a level in about 20 minutes which remained unchanged even after incubation for as long as 90 minutes.

Electron micrographs of spray preparations of particles incubated for 30 minutes in HCl and in pepsin are shown in Figs. 9 and 10, respectively. The air-dried HCl-treated particles are quite frayed and considerably flattened by comparison with untreated particles dried out of neutral buffer (Fig. 11). If allowance is made for this flattening, they appear to have about the same size as the untreated controls. Intermixed with the HCl-treated particles is seen some small heterogeneous particulate material, presumably detached from them. This small-sized material was never seen in preparations dried out of neutral buffer.

Air-dried preparations of virus particles which have been treated with pepsin (Fig. 10) have still another appearance. They are flattened in many cases, but they are less frayed and are considerably smaller in diameter than the HCl-treated particles. They do not have any small, particulate debris intermixed with them and, in fact, they seem fairly homogeneous in size when allowance is made for the varying degrees of flattening exhibited. Apparently, pepsin digests away that
exterior part of the original virus particles which is presumably denatured but only partially disrupted by the HCl (as well as the heterogeneous, detached material) and leaves behind an inner particle, or core. The homogeneity of size of these cores suggests that they are resistant to digestion by pepsin, a conclusion strengthened by the observation that incubation times up to 90 minutes do not change their appearance.

The least flattened of the core particles were found to have a diameter of about 90 mÅ, a value quite like that of the virus cores seen in the pepsin-treated sections. In view of this similarity in size, and in behavior when treated with pepsin, it is reasonable to believe that the cores obtained by digestion of whole particles are identical with those seen in the sections.

Chemical Analysis of the Peptic Digest

Ultraviolet absorption spectra of the core particles, the solubilized material representing digested outer shell, and the whole peptic digest of entire TIV particles are shown in Fig. 12. The core particles have a maximum absorption at 265 mÅ, quite close to the wave length of maximum absorption for the whole digest, 266 mÅ. The solubilized material, on the other hand, absorbs most strongly at 276 mÅ. This suggests that the core particles still retain a major fraction of the nucleic acid of the intact TIV particles and that the outer shell material released by digestion is mostly protein. This interpretation is supported by the values of the relative amounts of nitrogen and phosphorus in the 3 preparations, shown in Table I. The outer shell material released from the TIV particles by pepsin contains almost 3% of the nitrogen of the virus, but only 15% of its phosphorus; the core particles contain only 5% of the original nitrogen but at least 50% of the phosphorus. Clearly, all or nearly all of the nucleic acid of the virus must be in the core particles, and the digested peripheral material can be little else but protein.

The small amount of solubilized phosphorus not found in the core particles might possibly result from slight acid hydrolysis of nucleic acid during incubation with pepsin in 0.05 N HCl for 30 minutes. After prolonged incubation (120 minutes) it was, in fact, possible to demonstrate some release of nucleotides by a shift in the absorption peak of the soluble material to lower wave length. On the other hand, the solubilized phosphorus could easily arise from material other than the nucleic acid, since phosphoprotein accounts for almost 6 per cent and phospholipid more than 14 per cent of the total phosphorus in TIV (4).

From the data in Table I, the molar nitrogen : phosphorus ratio for the core particles is calculated to be 9.5. This is much higher than the ratio for TIV nucleic acid which is 3.7 (4), hence the core particles must contain protein as well as nucleic acid. The nitrogen content of both the nucleic acid and protein of the whole virus is about 17 per cent, and the known content of non-nucleoprotein material (lipid) is 5 per cent (4). From these figures and the nitrogen : phosphorus ratios, it can be estimated that the nucleic acid content of the core particles is 25 to 34 per cent, and their protein content is 60 to 65 per cent, the exact figures depending on what assumptions are made regarding the amounts of phosphoprotein and phospholipid in the core particles and the nature of the soluble and the unaccounted-for phosphorus.

DISCUSSION AND CONCLUSIONS

The protein nature of the outer shell, and the nucleic acid-plus protein nature of the inner core...
Absorption spectra of whole digest (a), core particles (b), and solubilized material (c) from peptic digestion of TIV. The whole digest and core particle preparations represent a TIV concentration of 0.114 mg/ml. The solubilized material represents a 3-fold higher concentration.

Absorption spectra of whole digest (a), core particles (b), and solubilized material (c) from peptic digestion of TIV. The whole digest and core particle preparations represent a TIV concentration of 0.114 mg/ml. The solubilized material represents a 3-fold higher concentration.

of TIV, here demonstrated directly by chemical analysis of isolated fractions, are consistent with the observations of enzyme attack on both whole and sectioned virus particles. Pepsin, quite specific for peptide bonds, attacks the outer shell, whereas trypsin, also specific for peptide bonds, together with DNase, specific for phosphodiester bonds, attacks the core of the particles when it is exposed by sectioning.

Since the entire core material may be washed from the sections with water only after both trypsin and DNase are applied, it seems likely that both the protein and the DNA are distributed throughout the core, very possibly as a nucleoprotein complex. If this complex exists, it would follow that the action of one enzyme alone would not effect sufficient degradation of the complex as a whole to render it soluble in water.

It is not surprising to obtain chemical evidence for a nucleoprotein composition of the viral core when one begins to relate the size of the core to the known (4) DNA content of the virus. The core is about 90 m\(\mu\) in diameter and the entire icosahedral virus has a minimum diameter of 130 m\(\mu\); consequently, the core has about 30 per cent of the volume of the virus particle. Its relative mass will be somewhat greater than this because of the greater density of its nucleoprotein content, but should be no more than about 33 per cent.

But the DNA of the virus accounts for only 12.4 per cent of its mass. Consequently the core must contain as protein about 20 per cent of the mass of the virus or about twice as much protein as nucleic acid. As we have seen, the nitrogen: phosphorus ratio of the core indicates twice as much protein as nucleic acid.

The fact that pepsin does not effect hydrolysis of the whole core material is probably explained, as is the similar failure of trypsin alone, by the protective role of the DNA in the presumed

| TABLE I |
| Relative Amount of Nitrogen and Phosphorus in Whole Peptic Digest, Core Particles and Solubilized Material |

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Nitrogen*</th>
<th>Phosphorus</th>
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<tbody>
<tr>
<td></td>
<td>Per cent of whole digest value</td>
<td>Per cent of whole digest value</td>
</tr>
<tr>
<td>Whole TIV digest</td>
<td>0.177 (100)</td>
<td>0.0160 (100)</td>
</tr>
<tr>
<td>Core particles</td>
<td>0.062</td>
<td>0.0143</td>
</tr>
<tr>
<td>Solubilized material</td>
<td>0.112</td>
<td>0.0008</td>
</tr>
<tr>
<td>(Sum of core particles and solubilized material)</td>
<td>0.174</td>
<td>0.0151</td>
</tr>
</tbody>
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* Values for whole digest and solubilized material are corrected for ammonium bicarbonate and pepsin nitrogen.
† A slight amount of the core particle pellet could not be resuspended, and was discarded. This probably accounts for the incomplete recovery of phosphorus.
nucleoprotein complex. There would seem to be no mystery about the failure of trypsin to digest the outer protein shell of the virus since it is presumably in a native configuration and insusceptible to proteolytic enzymes. Pepsin did effect hydrolysis, but only in the presence of 0.05 N HCl which at this concentration is a protein denaturant.

In demonstrating that TIV particles consist of a nucleic acid–containing core enclosed in a protein shell, the present experiments provide a new, clear-cut example to support what is coming to be regarded as the ubiquitous model for the structure of isometric ("spherical") viruses. There is currently little doubt but what this model represents most "spherical" viruses, but it should be pointed out that it has been actually based on direct chemical-morphological evidence for only a short list of examples; e.g., vaccinia virus (14, 15), and, less definitively, Rous sarcoma virus (16) and adenovirus (17). Inferential evidence supports the model in 2 other cases: turnip yellow mosaic virus (18, 19) and rabbit papilloma virus (20) where the presence of electron-microscopically "hollow" particles can be correlated with those that are devoid of nucleic acid. In a few other cases, such as poliovirus (21), herpes virus (22), and influenza virus (23), electron micrographs of transected particles disclose an inner core of greater opacity; this region has been assumed to contain nucleic acid. That this type of evidence may not be reliable, however, is demonstrated by influenza virus for which the DNA content (1 per cent) is far too small to account for the size and opacity of the observed core.

Besides showing that the nucleic acid of TIV is localized in its visibly distinct core, the experiments reported here demonstrate directly that the core contains a considerable amount of protein which is closely associated with the nucleic acid and probably exists as nucleoprotein. This observation helps to refine the simple concept of "spherical" virus structure, which, for lack of information, has not been explicit on the question of what else, if anything, exists in the core other than nucleic acid. It would seem, however, that some "spherical" viruses, for example, that of myeloblastosis (24, 25), must contain protein in their visibly distinct cores simply because, as with TTV, the amount of nucleic acid in the virus is not sufficient to fill up the volume of the core. In fact, at the present time there is no evidence, for any virus, that its nucleic acid exists in a distinctive region and in a pure state.

The technique used in the present experiments, digestion of sections with proteases and nuclease, may have application to the problem of electron microscopic identification of particles seen in sections of cells and presumed to be viruses. Some cytochemical information, such as the precise localization of nucleoproteins in sections of cells, should help immensely in discriminating viral from non-viral objects. The technique, as described here, would have to be improved in at least 2 respects to be wholly satisfactory: (1) retention of the embedding medium within the sections in order to maintain the optimal preservation of cellular ultrastructure, and (2) improved fixation which would preserve structure but would still allow the action of enzymes to take place. Osmium tetroxide, for example, prevents enzymatic action, while cold ethanol, which allows it, is a poor cellular fixative. There are some indications that these 2 improvements may be accomplished fairly readily. Experiments too preliminary to report in the body of this paper have shown that the cores of thin-sectioned TIV particles can be partially digested away even when the embedding methacrylate is retained in the section. (See Figs. 13 and 14). Epstein (16) and Epstein and Holt (17) have recently reported that in pellets of purified Rous virus and adenovirus the cores of the particles can be attacked by nucleases after fixation with KMnO4.

During the preparation of this manuscript a paper has appeared (26) in which it is shown that trypsin and pepsin can be used to digest differentially the structures of adenovirus and polyoma virus as they occur in sections of infected cells. The material was fixed with formalin and the embedding material was retained during enzymatic treatment of the sections. A water-soluble epoxy resin (Aquan) was used as embedding material; this may have facilitated the penetration of enzymes into the sections.

We wish to thank Dr. K. M. Smith for his hospitality in allowing one of us (Robley C. Williams) to prepare the virus material in his laboratory.

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FIGURE 13
Section of alcohol-fixed pellet; methacrylate retained, but treated with a 1:1 mixture of trypsin and DNase as for Fig. 7. Holes in the section are regions where virus cores have been removed.

FIGURE 14
Similar to Fig. 13, but treated with buffer only. The surface of the section shows the effects of knife impact, but the transected particles appear intact.