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THE MORPHOLOGY OF VIRUS-ANTIBODY INTERACTION

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I. Introduction

A. Background

In terms of the information it has yielded, the relationship between viruses and the electron microscope must be one of the most profitable of the century. Since the microscope became a practical tool during the 1930's, electron microscopy in general has benefited from techniques developed for the study of viruses in particular. Virology, in its turn, has progressed enormously as morphology has become available as an important aid to the better understanding of virus particles. The technique of shadow-casting was developed so that greater contrast could be obtained with virus particles, thin sectioning was advanced by the need to obtain better resolution on virus particles within cells, and the potential of the negative staining technique was only fully appreciated when it was applied to the study of virus fine structure. It is therefore not surprising that the field which we shall be discussing here, and which may be termed immune electron microscopy, was first developed specifically for visualizing the interaction of antibody molecules with virus particles.

Employing the technique of negative staining, immune electron microscopy offers information on various different aspects of the virus-antibody interaction and, since the background provided by negative staining lies beyond the resolving power of present day microscopes, the

instrument can be used to full potential, thus making it possible to recognize individual antibody molecules as such and also to distinguish between 7 S (IgG) antibody and the larger 19 S (IgM) antibody. In addition antigens can be located with accuracy within a larger structure such as a virus particle because the attachment of the antibody molecule to its corresponding antigen is actually visualized. Since the antibody molecule itself is visualized it is possible to carry out morphological studies both on the complete molecule and on the products of enzyme digestion. More recently it has been shown that it is possible to study not only the simpler systems with antibody and virus alone, but also the three component interaction between virus, antibody, and complement.

B. Historical

The first virus to be seen in the electron microscope in 1939 was that of tobacco mosaic. At this time the microscope itself was still in the developmental stage and such contrast as was obtained in these early micrographs was due simply to the small difference in density between the supporting film where no virus was present and the particles themselves. It is therefore a matter of wonder that under these conditions Anderson and Stanley (1941) carried out an electron microscope study on the reaction between tobacco mosaic virus and its antibody, and were able to show that meaningful results could be obtained with this approach. They found that there were demonstrable morphological differences between particles treated with specific antisera and those treated with control or nonspecific sera; they also went on to discuss aspects of lattice formation. This work was carried out some 30 years ago, and although we have microscopes with much better resolving power and techniques capable of providing greatly increased contrast, it is only now that we are beginning to take these pioneering studies a stage further.

The next attempt to use the electron microscope for the direct visualization of antigen-antibody complexes came when the technique of shadow-casting was developed (Williams and Wyckoff, 1946; Easty and Mercer, 1958, Hall *et al.*, 1959; Kleczkowski, 1961). This technique is valuable in that it allows three-dimensional studies to be carried out with the electron microscope; but it has the severe limitation that, because metal is evaporated onto the surface of the specimen, the resolution obtained can never be better than the granularity of the metal used. Under optimum conditions this resolution will be in the region of 25 Å (Bradley, 1961), a value that is not good enough when the molecules to be examined have dimensions of the same order. In addition the technique of shadow-casting requires that the specimens be dried down onto the grid and placed under vacuum before the metal can be evaporated

onto the surface; such a procedure cannot but be traumatic for the delicate three-dimensional structures formed by antigen and antibody.

The next attempts to visualize the immune process occurred when the technique of negative staining had become established as a means of elucidating the fine structure of viruses. This method was introduced by Brenner and Horne (1959) and overnight revolutionized the electron microscopy of viruses. Unlike shadow-casting the contrast is obtained by mixing the particles with a solution containing a heavy metal, generally phosphotungstic acid (PTA), which on drying down yields a more or less amorphous appearance and provides the contrast necessary to visualize the fine structure of the virus. In addition it appears from other work (Almeida *et al.*, 1966) that, when dry, the particles are surrounded and supported by a protein-phosphotungstate complex that will help to retain the original form of the particle or complex to be studied in a way that could never be achieved with shadow-casting. Its greatest importance in the present context is the fact that the method allows high resolution examination of the surface of particles and therefore of any structures attached to the surface. More specifically, if the virus has been exposed to antiserum, any antibody molecules attached to the surface will be visualized.

As frequently happens this approach to the visualization of the antigen-antibody complex, which we describe as immune electron microscopy, was exploited simultaneously but independently by two separate groups of research workers. These were Lafferty and Oertelis (1961) and Anderson *et al.* (1961). Lafferty and Oertelis used influenza virus for their antigen and were able not only to show distinctive changes on the virus projections when antibody was present but also to resolve individual antibody molecules. Anderson and his associates used mixed preparations of poliovirus and bacteriophages and showed that the particles, which are easily distinguishable morphologically, were aggregated only by their specific antisera. These experiments showed conclusively that the negative staining procedure could be used for selected types of immune reaction, and that the changes in the appearance of the virus particles after treatment with immune serum were the specific consequence of interaction with antibody.

II. Methods

In outline the technique is an extremely simple one, and frequently the greatest difficulty lies in obtaining a suitable virus suspension for use as antigen. (In this article it will frequently be found that the terms virus and antigen are given equivalent status. This is a matter of convenience,

and of course "antigen" in this context means antigenic sites located on the virus.)

One of the great values of the technique is that it can establish the occurrence of an immune reaction which it may be impossible to demonstrate in any other way; but paradoxically this does mean that it is frequently impossible to assess the relative titers of antigen and antibody, making it impossible to state categorically the amounts that must be used. However, it can be pointed out that immune electron microscopy is one of the most sensitive methods available for establishing the occurrence of an immune reaction and as little as 10^6 virus particles are sufficient to yield suitable specimens.

A simple procedure is to form the immune complexes from as high a concentration of virus as possible and to react this with a suitable fixed volume of antiserum undiluted, and at dilutions of 1:10 and 1:100. For example, using wart virus, for which there is no biological assay and which is obtainable only in such limited amounts that techniques such as complement fixation are usually too wasteful, 0.2 ml of a suspension containing approximately 10^8 virus particles per milliliter was mixed with 0.7 ml of physiological saline and 0.1 ml of the antiserum to be tested. After thorough mixing this was held at 37°C for 1 hour and then left overnight at 4°C. The following morning the complex was spun at 10,000 rpm for half an hour and the pellet was used for negative staining. The speed at which the antigen-antibody aggregate is spun must of course depend on the size of the virus, e.g., poliovirus plus antibody would need approximately 1 hour at 15,000 rpm, whereas a vaccinia-antibody complex can be spun down with ease in a clinical-type bench centrifuge. The much smaller centrifugal force needed for spinning down the complex as compared to the individual virus can be used as a means of improving the virus-to-background ratio with difficult viruses. For example, it allows rubella virus to be sedimented at speeds which are ineffective with the untreated virus.

It is important that the serum used for the reaction should not contain clumped protein, which can considerably obscure the final picture. This can be avoided by spinning the serum alone at 40,000 rpm for 1 hour before mixing it with the virus. In certain instances it is necessary to use not the whole serum but purified fractions of it, and this is mentioned in the text when it has occurred. However, for ordinary purposes it is necessary only to ensure that the antiserum is heat-inactivated so that complications arising from the presence of complement do not occur.

In some cases, after the immune complex has been spun, examination in the electron microscope will reveal that the aggregates are considerably obscured by the presence of low molecular weight protein derived

either from the serum or from the virus preparation. It is then advisable to resuspend the complex in the original volume of phosphate-buffered saline and recentrifuge at the speed used previously. This treatment can rescue a bad specimen and may considerably improve what had been a reasonably good one.

Negative staining is carried out in a routine manner as follows. The final pellet is resuspended in a small volume of distilled water (approximately 0.1 ml) and a drop of this is mixed immediately with an equal volume of 3% PTA adjusted to pH 6. A drop of this mixture is then placed on a 400-mesh carbon-Formvar-coated grid, excess fluid is withdrawn with filter paper, and the grid is placed immediately in the microscope. It is important that the time lag between suspending the pellet in distilled water and placing the grid in the microscope be kept as short as possible, because conditions are nonphysiological at this time and dissociation or other changes will almost certainly take place.

III. Results

A. Basic Findings

The negatively stained appearance of virus particles is now well known. The electron-dense PTA surrounds the particle rather than penetrates it, and contrast is obtained simply by the fact that the electron-scattering ability of the virus particle is low compared to the background of PTA and is seen as a hole in the dense substrate. However, since the PTA appears amorphous it is possible to delineate not only the overall outline but also the detail on the surface of the particle. By this means many viruses have been shown to display a definite symmetry either on the surface or in an internal component. Even when symmetry has not been shown other distinctive features are present on the virus particle, for example, the projections on the surface of coronaviruses (Almeida *et al.*, 1968) which allow them to be recognized with ease. The most obvious effect of antibody is the obscuring of this clear-cut and distinctive fine structure. This, together with clumping, produces an unmistakable effect so that antibody-treated virus presents a very different picture from that of the untreated control. Figures 1 and 2 show the appearance of wart virus particles before and after they have been reacted with specific antiserum. In Fig. 1 the particles display sharp outlines and it is possible to observe the arrangement of subunits forming the capsid. It should also be noted that although there is a high concentration of virus in this micrograph the particles are randomly distributed throughout. In Fig. 2, on the other hand, the particles are obscured and even appear fuzzy, although the micrograph would be considered as showing good

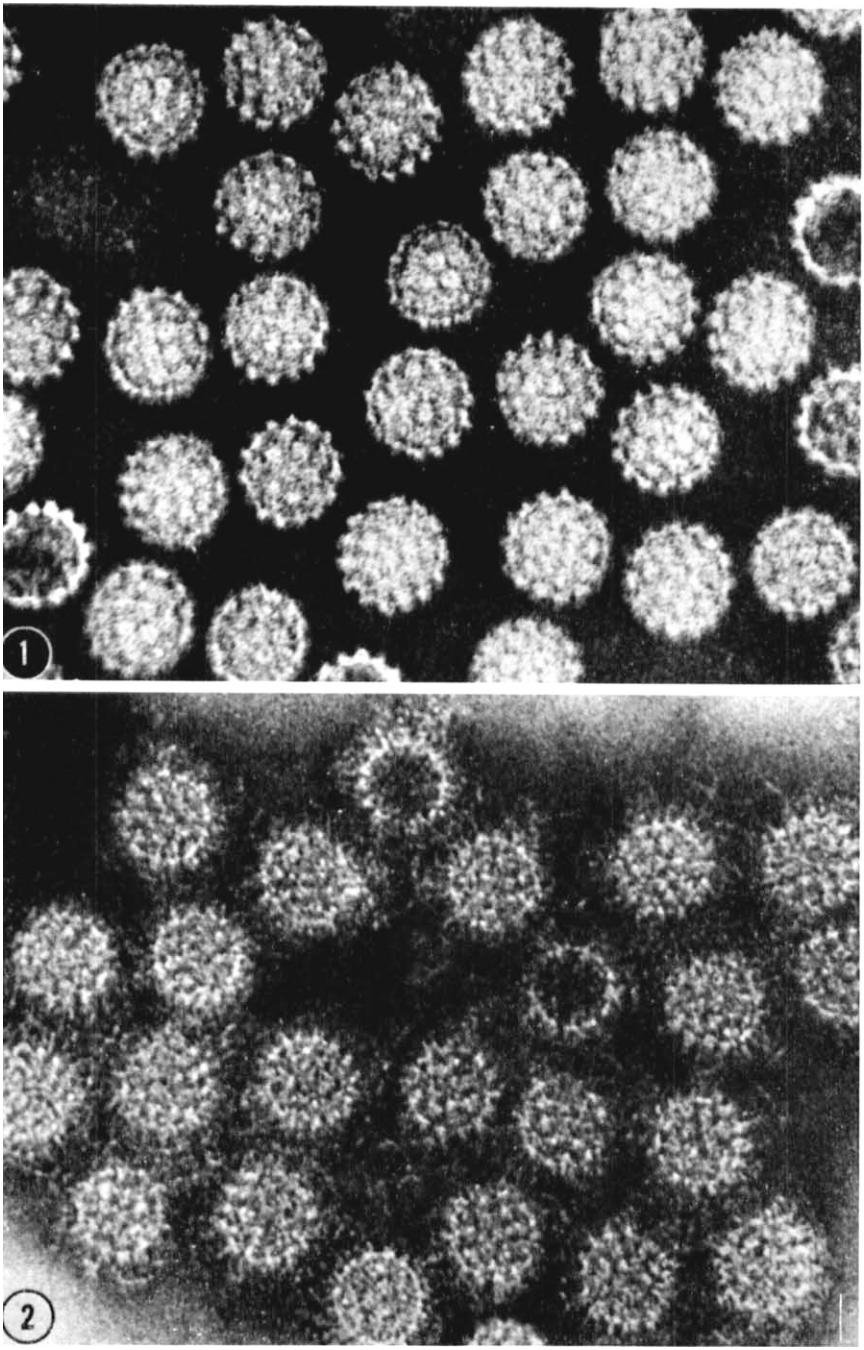


FIG. 1. A control preparation of human common wart virus particles as they appear with negative staining. The subunit construction of the virus is clearly delineated and its distribution can be seen to be random. Magnification × 300,000.

FIG. 2. The same preparation of virus as is shown in Fig. 1 after treatment with specific antiwart virus antiserum. The particles are now obscured by the presence of linear molecules which appear around the virus as a halo; clumping has occurred. Magnification × 300,000.

resolution. The halos surrounding the particles can be resolved in places into individual linear structures, and it should be noted that the particles are no longer randomly distributed but clumped within the center of the micrographs. This phenomenon of clumping, although it can be illustrated by single micrographs, is much more impressive when actually seen in the microscope, as very few single particles remain and examination of such a grid reveals only aggregates, ranging from those containing a few virus particles to others which are so large and dense that no proper resolution can be obtained with them (Fig. 3). The phenomenon of clumping can be both recognized and recorded at low magnification, but to obtain detail of the antigen-antibody interaction it is necessary to work at a magnification of 40,000 or greater.

That these changes are both specific and due to the attachment of antibody molecules was first shown in 1962 by Hummeler *et al.* with poliovirus. Heat-inactivated (H) poliovirus particles appear with negative staining as hollow structures having a distinct rim (so-called ring staining). Native (N) virus, on the other hand, appears full with no rim around the edge. Using specific anti-N and anti-H antiserum these workers showed that the aggregates in each case were composed of particles having the expected morphological form of the group. Shortly after this two other groups of workers also showed that the changes seen in the electron microscope were specific ones. Almeida *et al.* (1963) used mixtures of wart and polyoma virus, which are similar in appearance but slightly different in size. To this mixture of viruses was added antiserum to polyoma virus alone, and it was only the smaller particles that became obscured and clumped by the presence of antibody molecules (Fig. 4). Lafferty and Oertelis (1963) used influenza virus as their system, and showed that influenza A virus changed markedly in appearance after the addition of influenza A antiserum, while the morphologically identical influenza B virus remained unaltered after the addition of the same antiserum but showed a positive reaction after the addition of antiserum to influenza of the B group.

After the technique had in this way proved its value in the direct identification of specific antigens it was adapted to various aspects of the interaction between virus and antibody (Watson and Wildy, 1963; Bayer and Mannweiler, 1963), and the technique was extended for use with nonviral antigens of a size suitable for negative staining (Elek *et al.*, 1964; Feinstein and Munn, 1966; Valentine and Green, 1967).

The ease with which antigen-antibody aggregates could be visualized meant that several different aspects of such interaction could now be studied directly and in a way which had formerly been impossible. Such aspects included lattice formation, the morphology of the antibody mole-

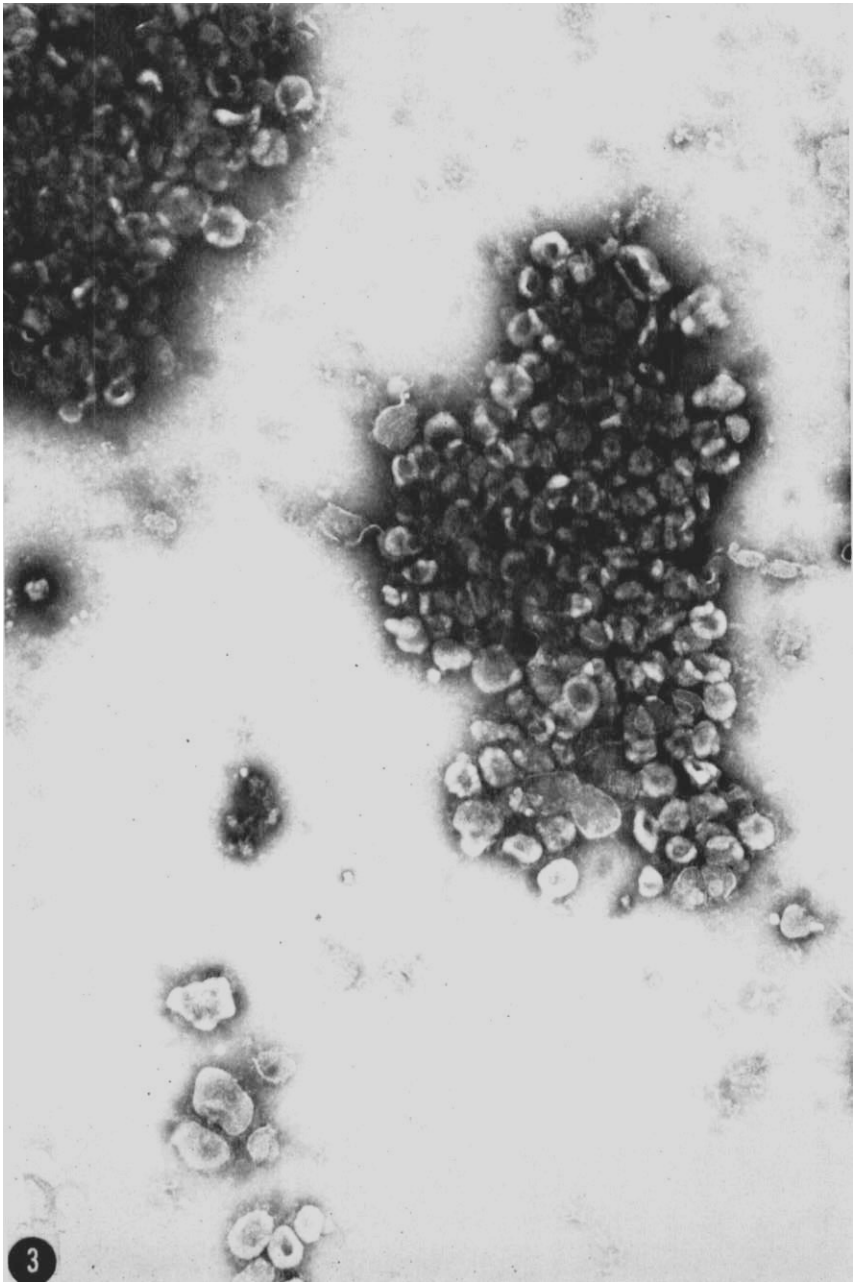
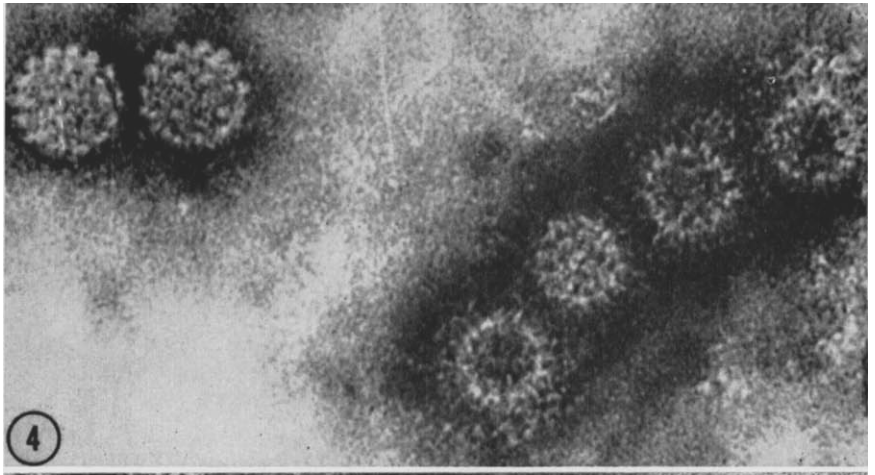


FIG. 3. A low-power micrograph of an avian infectious bronchitis virus and antibody preparation. This is to illustrate more clearly the phenomenon of clumping. Most of the grid square appears empty, almost all the virus being concentrated in one of the two large clumps present in the field. Magnification × 70,000.



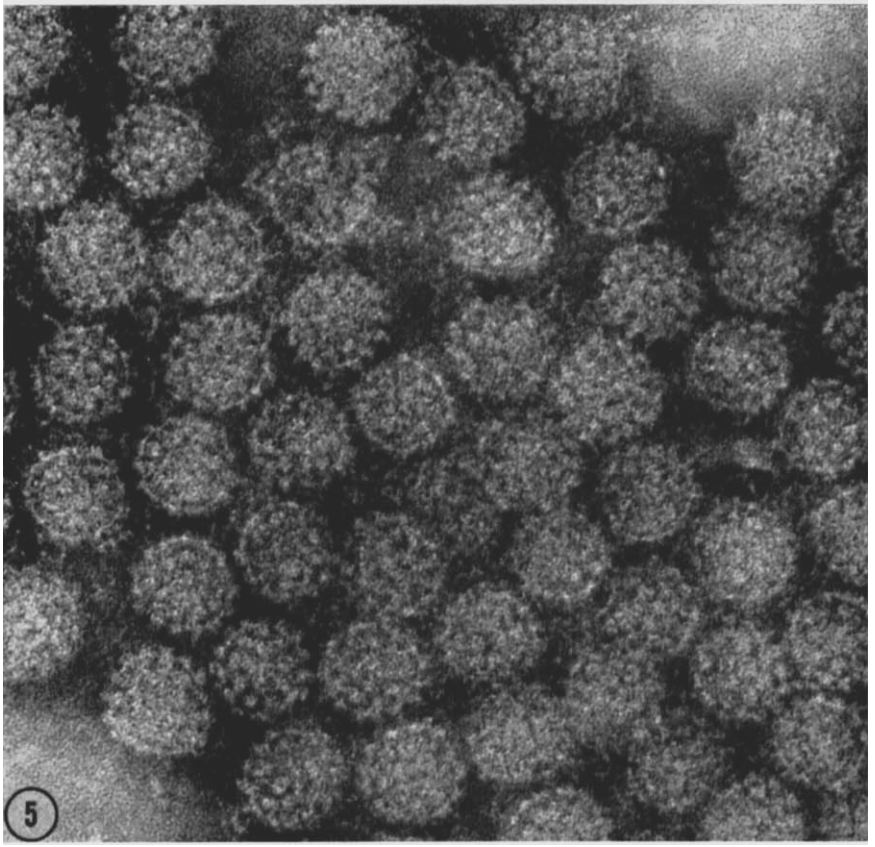


FIG. 4. That the changes visualized in the electron microscope are specific ones is shown in this micrograph. Polyoma antiserum was added to a mixture of wart and polyoma viruses. Only the slightly smaller polyoma particles are obscured and aggregated by antibody molecules. × 300,000. FIG. 5. An aggregate of wart virus particles and antibody from human sera. Such micrographs confirmed that lines obtained in agar gels were the result of interaction between virus and antibody. × 300,000.

cules themselves, the appearance of enzyme-treated antibody, and the difference in appearance between the two component antigen-antibody system and the three component antigen-antibody-complement system. Also, and this is by far the most direct use to which the method can be put, it is possible to verify visually what components are taking part in an antigen-antibody reaction. These several aspects will now be considered under separate headings starting with the last stated, the identification of the antigen.

B. Identification of Antigens

It frequently happens with virus systems that it is possible to show the presence of an immune reaction, but, because the virus-antigen preparation itself is far from pure, it may prove difficult to show that the reaction is specifically between the virus and antibody. In our own laboratory we found a problem of this type with wart virus systems for which there is no biological assay. In this case immunodiffusion showed that precipitin lines could be obtained with the sera of roughly 50% of patients with warts, but since neutralization assays were not possible the only way to show that the antigen component of the line was virus was to examine the antigen-antibody mixture in the electron microscope, with the result, in this particular case, that the reaction was shown to be wholly dependent on the virus particles (Fig. 5) (Almeida and Goffe, 1965). In this example we examined a parallel preparation to the one that had been used for immunodiffusion. But this approach can be taken a step further and if the immune reaction has been established by means of the agar gel immunodiffusion technique it is possible to cut out the precipitin lines and use them for negative staining thus leading to positive identification of the components taking part in the reaction. Watson and Wildy (1963) used this method for the identification of herpes capsids, and Beale and Mason (1968) were able to show which precipitin line in a poliovirus-agar gel system belonged to the H and which to the C component.

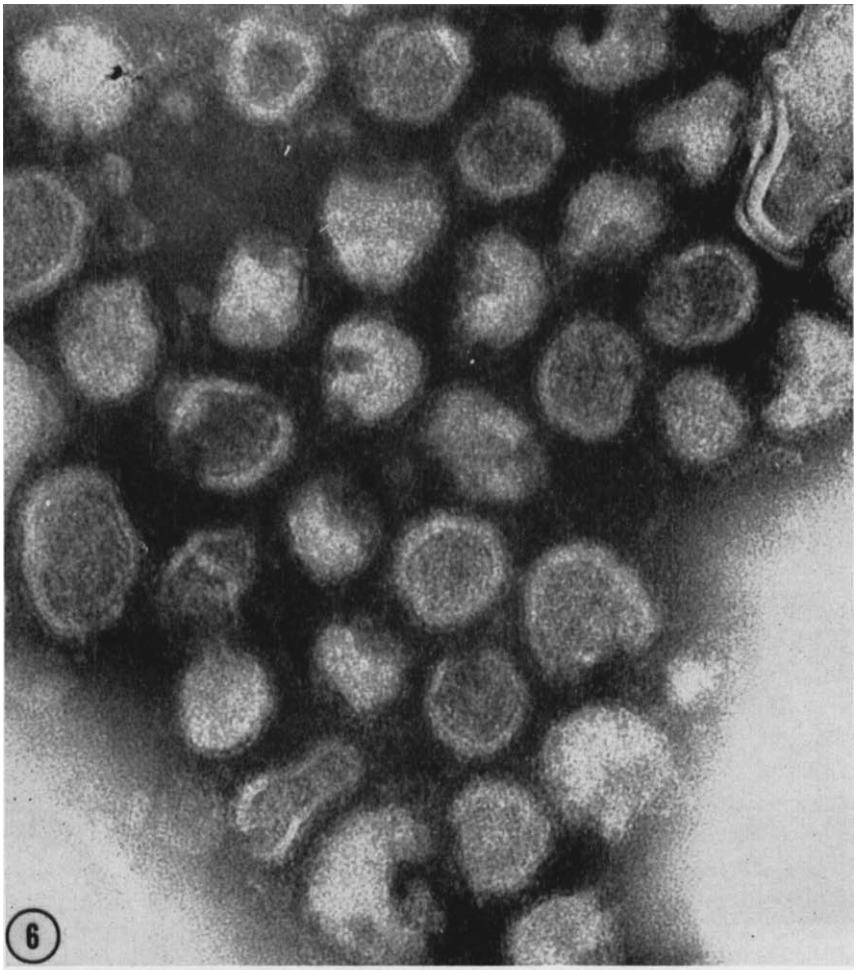
Another use of the technique not directly concerned with the study of the immune reaction but concerned with the identification of antigens is its application to the study of viruses present only in low titer or which are difficult to identify with assurance. It frequently happens that the ratio of virus particles to background material cannot be changed because the contaminating debris is roughly the same size or density, or both, as the virus. Density gradient centrifugation may well be the answer in such cases, but frequently there is not enough material of sufficiently high titer for this technique to be feasible. However, if, the virus, after partial purification, is mixed with antiserum to produce aggregates

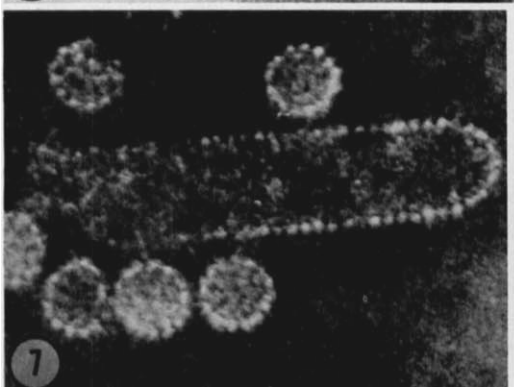
of virus it is then possible to spin at a much lower speed than would be necessary to sediment individual virus particles, thus leaving in the supernatant the low molecular weight material which is a much greater problem to electron microscopy than the large, easily recognizable debris. Using this approach we were able to identify with certainty for the first time the virus of rubella (Best *et al.*, 1967), an agent which had proved to be one of the most difficult of the human pathogens to characterize morphologically. The reason for this was only appreciated after the virus had been seen in large antibody-bound rafts which left no doubt about the identity of the virus for, individually, the particles exhibited none of the distinctive symmetry features usually associated with virus particles (Fig. 6). In this example the problem of centrifugation was a very real factor, as it is only with difficulty that individual rubella particles can be sedimented, whereas the rubella-antibody aggregates can be spun down at the relatively low speed of 12,000 rpm for 30 minutes in the SS 34 rotor of the Sorvall RC 2B centrifuge.

Another means by which antigens can be identified in the electron microscope is by making use of the fact that individual antibody molecules have identical binding sites at either end of the molecule (Nisonoff and Pressman, 1959) so that if a single antibody molecule can be seen attached at one end to a structure containing a known antigen and at the other to an unidentified antigen, one can presume that the identity of the two structures is proven. There is a possibility that a small population of heteroligating antibodies may be present in any one preparation, so the occurrence must be shown to be reproducible before being finally accepted. This approach has been used to show that the aberrant forms of wart and polyoma virus share at least some antigens with the standard icosahedral form of the same viruses. In Figs. 7 and 8 two examples of antibody molecules linking aberrant particles of polyoma to several normal particles can be seen.

C. Lattice Formation

The phenomenon of flocculation has long been a matter of immunological speculation (Bordet, 1920) and is here taken to include the formation of aggregates of simple molecular antigens as well as those on a grosser level. Probably the first realistic appraisal of the precipitin curve came from Marrack in 1938 when he put forward the lattice theory of immune complex formation. This theory was further developed in 1940 by Pauling, and the views put forward at that time, have, with slight modification, remained valid until the present day (Humphrey and White, 1964). The lattice theory offers an explanation as to why flocculation should occur around the region of equivalence, when antigen and





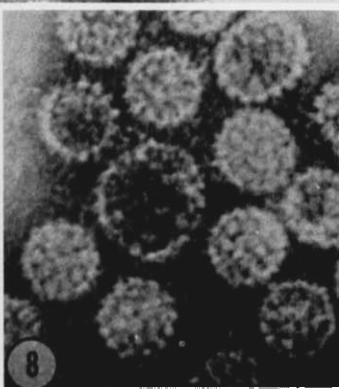


FIG. 6. Rubella virus has probably been one of the most difficult pathogens to characterize morphologically. Here it is seen combined with antibody, an approach that allowed it to be identified with certainty. Magnification × 250,000.

FIGS. 7 AND 8. These two micrographs show examples of aberrant forms of polyoma virus linked to normal particles by antibody molecules. Such linkage shows that the aberrant forms share at least some antigens with the standard virus. Magnification × 250,000.

antibody are present in optimal proportions with respect to each other, and as to why it should not occur to any great extent in either the regions of antigen or antibody excess (Fig. 9). In 1963 Almeida *et al.* discussed the results obtained from immune electron microscopy when wart and polyoma viruses were combined with antiserum in concentrations ranging from extreme antigen excess through to antibody excess. These showed a close similarity to the diagrammatic drawings of the lattice formation as developed by Pauling (1940). In the region of extreme antigen excess only a few antibody molecules could be seen attached to virus particles. Linking between virus particles could be seen to have occurred by small numbers of antibody molecules on one virus particle becoming attached by the free binding site at the other end of the molecule to another virus particle (Fig. 10). With rising antibody concentration, al-

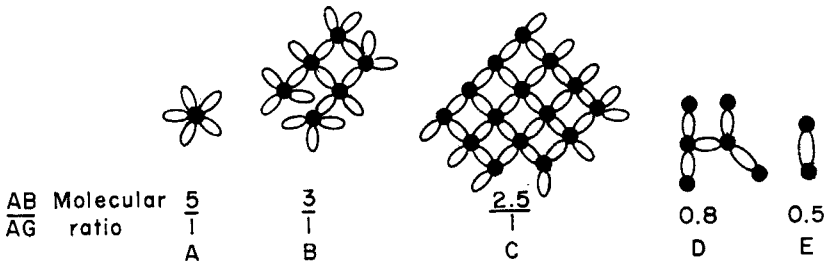
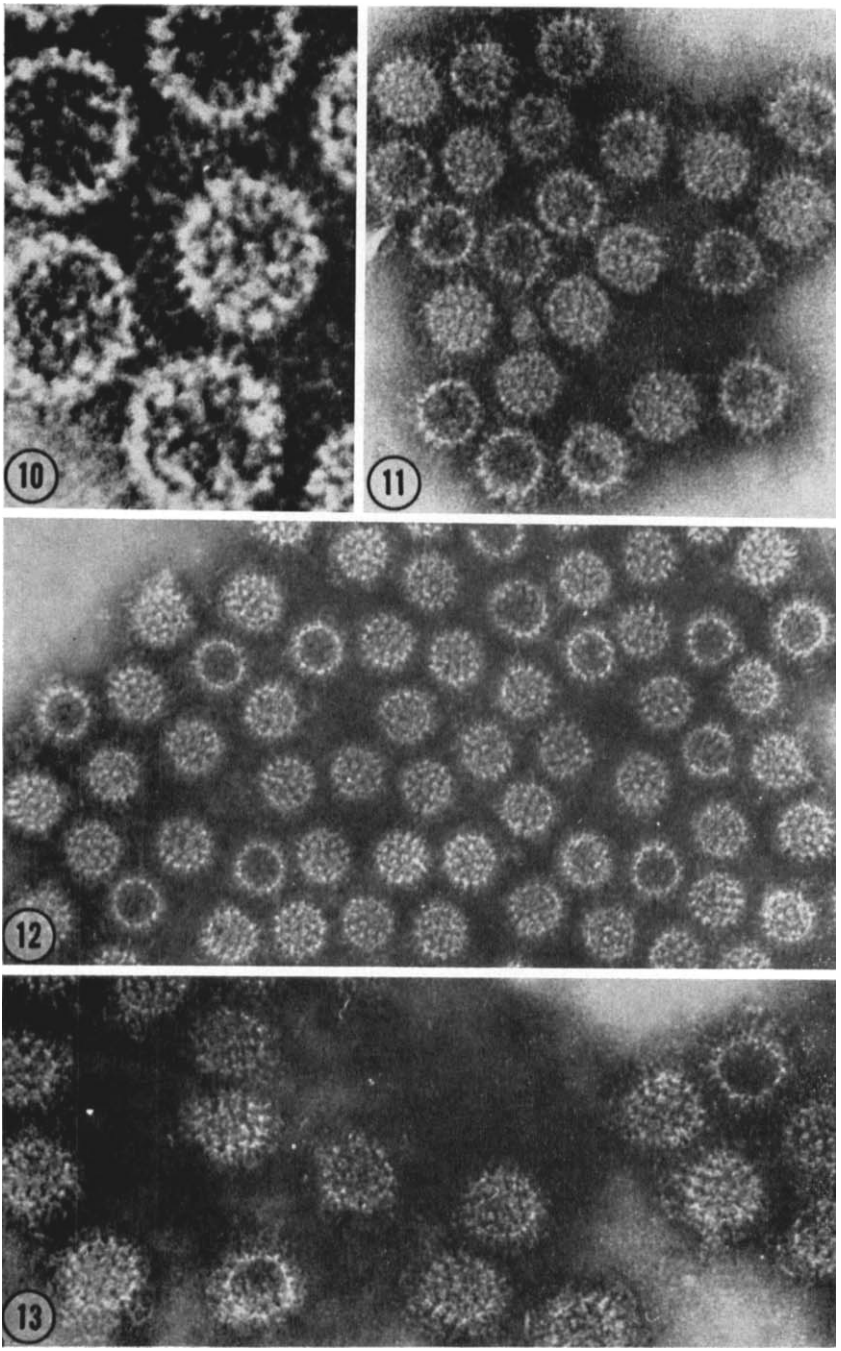


FIG. 9. A diagrammatic representation of possible arrangements of antigen (shaded) and antibody at different relative concentrations of antigen and antibody. (Humphrey and White, 1964.)

though still in the region of antigen excess, the size of these aggregates increased (Fig. 11). At and around the equivalence region the specimen consisted almost entirely of large rafts of antibody-linked virus (Fig. 12), most of which were too large and, because of three-dimensional build-up, too dense for useful photographic record. As the region of antibody excess was approached large aggregates remained but a change could be seen in their formation. Available antigenic sites on the virus particles were fully occupied by antibody molecules and hence cross-linking between particles by antibody molecules was no longer possible. The aggregation that did occur at this stage was due to entanglement of the antibody halos around separate virus particles (Fig. 13), and would seem to be dependent on the amount of centrifugation to which the complex had been subjected; if not centrifuged, no aggregation occurred. That two different kinds of linkage did occur on the antigen excess and antibody excess side of the curve was verified by measuring the distance between aggregated virus particles at different points on the curve. Anal-



FIGS. 10–13.

ysis showed that a statistical difference existed between the spacing of particles in regions of antigen and antibody excess.

These results would seem to confirm the theoretical speculations put forward for the formation of immune complexes at different relative concentrations of antigen and antibody. However, negatively stained immune complexes will always have certain limitations associated with them. Only those complexes built from suitably sized particles can be used. Viruses, most of which are in the range of 250 to 2000 Å, are ideal because structures either much larger or much smaller have technical problems attached to them. For example, macromolecules smaller than viruses may be difficult to distinguish from the antibodies themselves, while large structures such as bacteria tend to obscure the antibodies and yield poor resolution. Second, since the electron microscope is a transmission instrument it will always be necessary to select for examination those aggregates which are arranged more or less two dimensionally, as resolution decreases rapidly with increase in specimen thickness. Also, even though negative staining does not expose the specimen to the same severe effects as shadow-casting, distortion must occur during the drying down process, and it must be considered that small aggregates that appear two dimensional in the microscope may not have started that way.

D. The Morphology of Antibody Molecules

This section on morphology will be split up into a subsection dealing with IgG and one with IgM antibody; as yet, IgA antibody has not been visualized in the electron microscope.

1. IgG

Unless stated to the contrary the term antibody generally refers to the IgG component of the immune globulins. The antibody in hyper-

FIG. 10. At concentrations of antibody below equivalence small groups of particles are linked directly by small numbers of linear antibody molecules. Magnification × 500,000. (Figures 10–13 form part of a series in which polyoma and wart viruses were combined with differing concentrations of antibody.)

FIG. 11. Near equivalence the particles are still linked directly by antibody but the size of the groups is larger. Magnification × 170,000.

FIG. 12. This micrograph is from the region of antibody excess and although there is considerable aggregation many of the particles are no longer directly linked to each other by antibody molecules. Instead they are surrounded by halos of antibody occupying all binding sites and making cross-linking impossible. Magnification × 150,000.

FIG. 13. In extreme antibody excess there is no cross-linking between particles; each virus is enclosed in an antibody halo and any clumping that occurs is due to entanglement of the antibody molecules. Magnification × 180,000.

immune sera consists almost entirely of this species, which has a molecular weight of 150,000 and a sedimentation coefficient of 7 S. Although much is known about the chemical constitution of the molecules the exact morphology is still a matter of conjecture. Hydrodynamic data suggest that the molecule is elongated, and there is indirect evidence that the two binding sites on the divalent IgG molecule are located at some distance apart. However, the results are by no means conclusive, and recent reappraisal of the hydrodynamic data has led to the suggestion that the IgG molecule could also be interpreted as a Y-shaped structure (Noelken *et al.*, 1965).

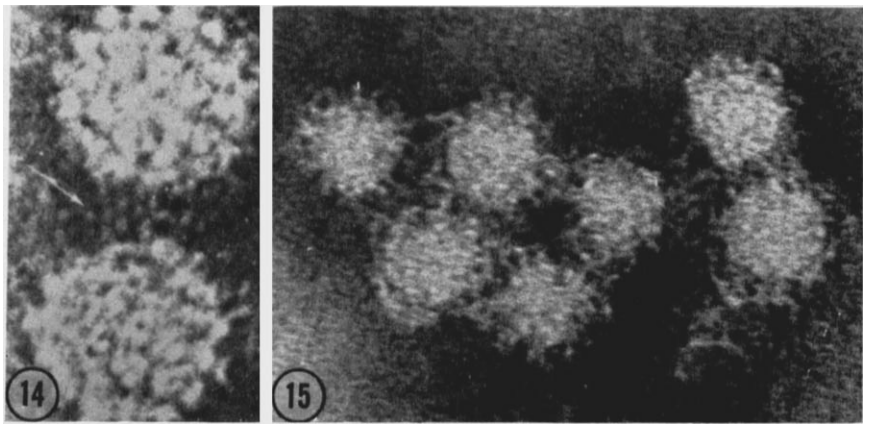


FIG. 14. Two wart virus particles linked by IgG antibody molecules which appear linear. Magnification × 550,000.

FIG. 15. A group of foot and mouth disease virus particles combined with IgG antibody. Once again the linear nature of the molecules can be seen and in addition this micrograph should be compared with Fig. 18 which shows the same virus combined with IgM antibody. Magnification × 500,000.

For the most part the results from negative staining have favored the elongated structure. In the 1963 paper by Almeida *et al.* micrographs of wart and polyoma virus showed linking antibody as linear structures of approximately 35–40 × 250 Å (Figs. 10, 14, 15). Binding sites were located at opposite ends of the linear molecule. Similarly, Lafferty and Oertelis (1963) reported that in their micrographs the binding sites appear to be located on or very close to each end of the antibody molecule. Elek *et al.* (1964) also report a linear appearance for IgG antibody molecules in a study with bacterial flagella.

However, in 1965 Feinstein and Rowe published micrographs of antibody molecules directed against ferritin in which they delineated a Y-shaped structure. This was followed in 1967 by a report from Valen-

tine and Green who also found evidence supporting the "Y" morphology when IgG was studied, using dinitrophenylpolymethylenediamine (DNP) as the antigen. With this system the antigen DNP is too small to be visualized by the negative staining procedure, so that it is possible to visualize combined antibody without antigen appearing in the micrograph. In order to understand their results it will first be necessary to consider briefly the proposed chemical structure of the IgG molecule. This has been reviewed by Fleischman (1966) and the molecule is described as a dimer (Fig. 16), each half of which contains an H (heavy) chain (molecular weight 50,000) and an L (light) chain (molecular weight 20,000). When digested with papain the molecule splits into three parts, two of which are identical and retain an antigen-binding site. These form the so-called "Fab" fragment which can be looked on as

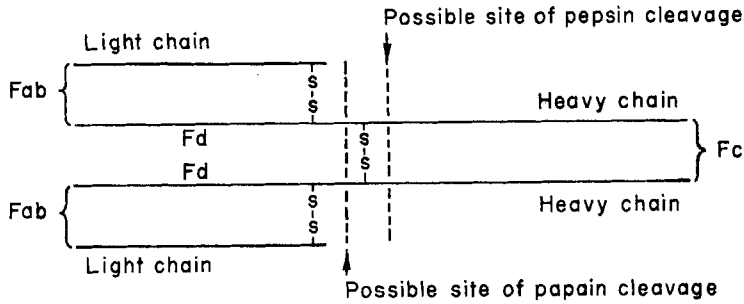


FIG. 16. Diagrammatic representation of one possible model of IgG immunoglobulin. (Fleischman, 1966.)

monovalent antibody. The third fraction has no combining activity, is of low molecular weight, comes from the central region of the complete molecule, and is referred to as the Fc fragment because under certain conditions it can be made to crystallize.

Both on micrographs and in a diagrammatic representation (Fig. 17), Valentine and Green show the IgG molecule as having the two Fab portions in the form of a V while the Fc fraction forms a leg, giving the whole structure a Y-shaped appearance. While the simple linear model yielded dimensions of 35 × 250 Å the Y model is in the range 35 × 150 Å. They also showed that after treatment with pepsin, which specifically digests the Fc fragment, the leg of the Y disappeared, adding considerable weight to their hypothesis. However, the Y-shaped molecule would seem to be somewhat of a paradox in at least one respect, for if the two combining sites are situated as suggested in Fig. 17, then it is surprising that so few antibody molecules combine with two antigenic sites located on the same virus particle. Undeniable looping of antibody

molecules, where the antibody had combined with two sites on the same virus particle, was found only rarely in our studies whereas linking between two different particles was the general occurrence (Figs. 10, 14, 15). It has been suggested in this context that antigenic structures such as viruses would exert a distorting effect on the antibody molecules, pulling them out so as to give a linear appearance which is artifact. The likelihood of distortion is very real, but one must still answer why the antibody molecule should have attached to sites on each of two different virus particles in the first place, as this would not occur unless the binding sites were located at some distance from each other on the antibody molecule. As we shall see a similar problem arises in the in-

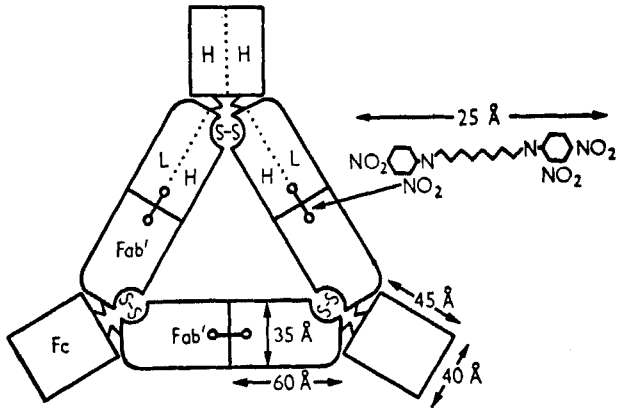


FIG. 17. Diagram illustrating the electron microscope findings of Valentine and Green (1967) with the DNP and IgG antibody system.

terpretation of the morphology of IgM antibody, and here again it appears that the nature of the antigen used can influence the appearance of the antibody structure obtained. It must also be observed that in studies on antibody structures we are using the electron microscope near the limit of practical resolution for biological material, even though the actual resolution is considerably better. This in turn means that it is dangerous to put too definite an interpretation on some of the structures visualized until a more solid basis of information has been obtained from a wider range of antigen-antibody systems.

2. IgM

This antibody is the first to be detected in a primary response to an antigen. It is a large molecule, with a molecular weight of approximately one million, and a sedimentation coefficient of 19 S. It can be distinguished from IgG antibody in that it can be degraded to smaller com-

ponents by breakage of disulphide bonds with reducing agents such as 2-mercaptoethanol. This cleavage of the molecule yields five fragments having a sedimentation coefficient of 7 S. Recent immunochemical data suggest that the IgM molecule has 10 binding sites but that they do not all have equal affinity for the antigen. (Merler *et al.*, 1968; Onoue *et al.*, 1968).

In order to study the IgM molecule in the electron microscope it is necessary for it to be purified, as it is invariably contaminated with the IgG molecule. Purification can be carried out by Sephadex gel filtration (Feinstein and Munn, 1966) or by sucrose density gradient centrifugation (Almeida *et al.*, 1967; Svehag and Bloth, 1967).

IgM was first visualized by Humphrey and Dourmashkin (1965) using sheep red cell membrane as the antigen. Red cell membrane fragments roll up into strips of suitable dimensions for negative staining, making it possible to resolve any structures attached to their edges. The IgM molecules appeared as distinctive "staple"-shaped structures with a clearly angular outline. This "staple" form was confirmed in 1966 by Feinstein and Munn using bacterial flagella as the antigen. In 1967 Almeida *et al.* reported a study of IgM using foot and mouth disease virus (FMDV) as the antigen (Fig. 18). They also confirmed the staple configuration (Fig. 19) but also found that where the antibody linked antigenic sites on different virus particles rather than sites on the same particle the molecule could appear linear (Fig. 20) with lengths of up to 370 Å and average dimensions of 350 × 50 Å. One of the most striking differences between IgG and IgM antibody molecules is the apparently greater flexibility of the latter, which may simply be a function of the greater length of this molecule. Looping, which was encountered rarely for IgG antibody, was present in all the micrographs obtained with IgM, and the staple form, which has been most frequently seen, is in fact a form of looping.

Once again, as for IgG, the appearance of the molecule has been considerably influenced by the type of antigen used. Sheep red cell membrane fragments and bacterial flagella, because of their relatively large size, have numerous antigenic sites available on a single structure so that probability will favor the attachment of both combining sites of the antibody to the same fragment. The FMDV preparation, on the other hand, was used at such a concentration that antigenic sites would be just as available on separate particles as on the same one. In addition, the particle of this virus, whose overall diameter is only 240 Å, can have only a very limited number of antigenic sites available for attachment, whereas red cell fragments and bacterial flagella are not only large but are also linear, two features which would facilitate attachment to the

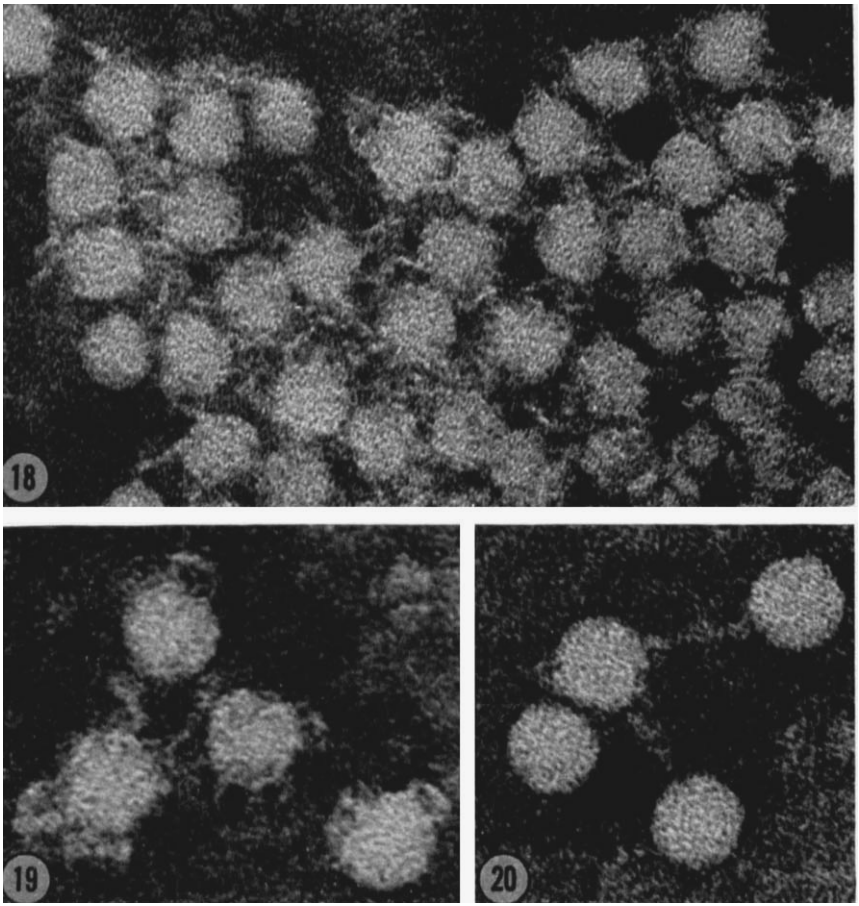


FIG. 18. A larger group of foot and mouth disease virus particles in combination with IgM antibody. The appearance of this complex is quite different from those formed with the lower molecular weight IgG globulin. Magnification × 420,000.

FIG. 19. Another group of foot and mouth disease particles combined with IgM antibody. Here the antibody molecules display the "staple" form of morphology which is found most frequently when larger antigenic structures are employed. Magnification × 500,000.

FIG. 20. A small group of foot and mouth disease virus particles three of which are linked by linear molecules of IgM antibody. These molecules are larger and more distinct than the IgG molecules shown at the same magnification in fig. 15. Magnification × 500,000.

same fragment. More recently Valentine (1968) has studied isolated IgM antibody and was able to show a starry structure with five points which would of course be a very acceptable form for a molecule with 10 proposed binding sites. The fact that this molecule seems to present

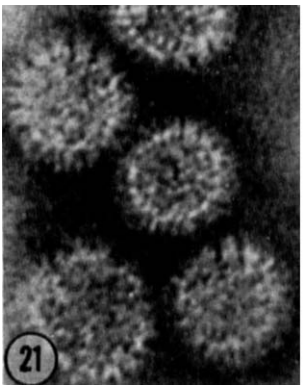
several different appearances in the electron microscope need not be due to artifact, as combination with antigen may well produce configurational changes in the molecule, changes which may, at least in part, be influenced by the size and type of structure bearing the determinants.

To sum up this section on antibody morphology, negative staining enables both IgG and IgM not only to be visualized with ease but also to be distinguished from each other. What it does not do is allow a final appraisal of the exact anatomy of these two molecules before and after combination with antigen. Further work with different antigenic systems will undoubtedly yield new information on antibody morphology, but it may well be that either a refinement of present day negative staining techniques, or a completely new approach, will be necessary before the resolving power of the electron microscope can be used to its full potential in this field.

E. Products of Enzyme Digestion

IgG antibody can be degraded in one of two ways. Using papain the molecule is split into two identical fragments retaining antigen-binding capacity (Fab) and a third noncombining fragment which under the correct conditions will crystallize (Fc) (Porter, 1959). With pepsin as the degrading agent the Fc fragment is split off but the two Fab fragments remain linked by a disulfide bond (Nisonoff *et al.*, 1960). The products of both of these digestion methods have been studied in the electron microscope and it has been possible to observe the changes in the IgG antibody molecule produced by them. Using polyoma virus as the antigen Almeida *et al.* (1965) studied the attachment of monovalent papain-digested antibody. The products of digestion appeared as rigid, radially oriented rods of approximately 70–90 Å (Fig. 21), whereas the complete divalent antibody molecule is seen around the virus as a halo of flexible, randomly oriented molecules of average length 250 Å. That the fragments did represent digested IgG molecules was shown by reacting the virus plus univalent fragments with goat antiserum directed against rabbit gamma globulin (the species used for preparing the polyoma antiserum). This changed the 90 Å halo surrounding the particles into one of 300 Å, while control virus treated with the same goat antirabbit gamma globulin antiserum showed no change at all. Feinstein and Rowe, also in 1965, looked at papain-treated antibody to ferritin molecules and obtained dimensions of 100 Å for the Fab fragments. Valentine and Green (1967), using the DNP system previously described, looked at both pepsin-digested and papain-digested IgG, and found it possible to distinguish between them. Their results are of course based on the Y-shaped interpretation of the IgG molecule. Using pepsin they found that only the

projection corresponding to the Fc fragment disappeared and that the overall morphology of the antigen-antibody complex was retained. However, with the use of papain the molecule was disrupted leaving small rodlike structures with dimensions of 60 to 80 × 35 Å. This means that all of the workers who have examined papain-digested IgG have found linear structures in the 60–100 Å size range.



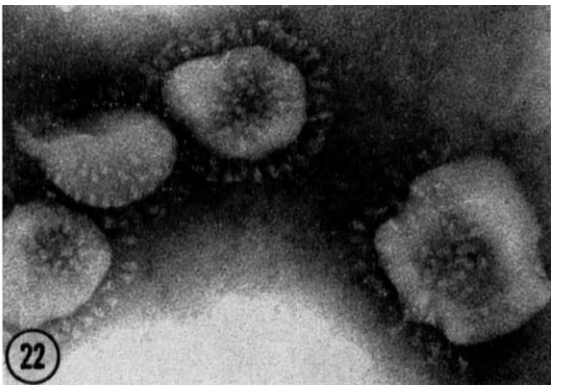


FIG. 21. This micrograph shows polyoma virus with adsorbed univalent antibody. The IgG molecules had been digested with papain and it is the fragment described as Fab that is illustrated. The fragments are approximately 70 Å long and appear much more rigid than the complete IgG molecule. Magnification × 300,000.

FIG. 22. This micrograph illustrates the appearance of control avian infectious bronchitis (IB) virus. The particles are pleomorphic and have as their outstanding feature petal-shaped projections approximately 200 Å long. As can be seen on the particle on the right there are areas from which the projections have been lost. Magnification × 200,000.

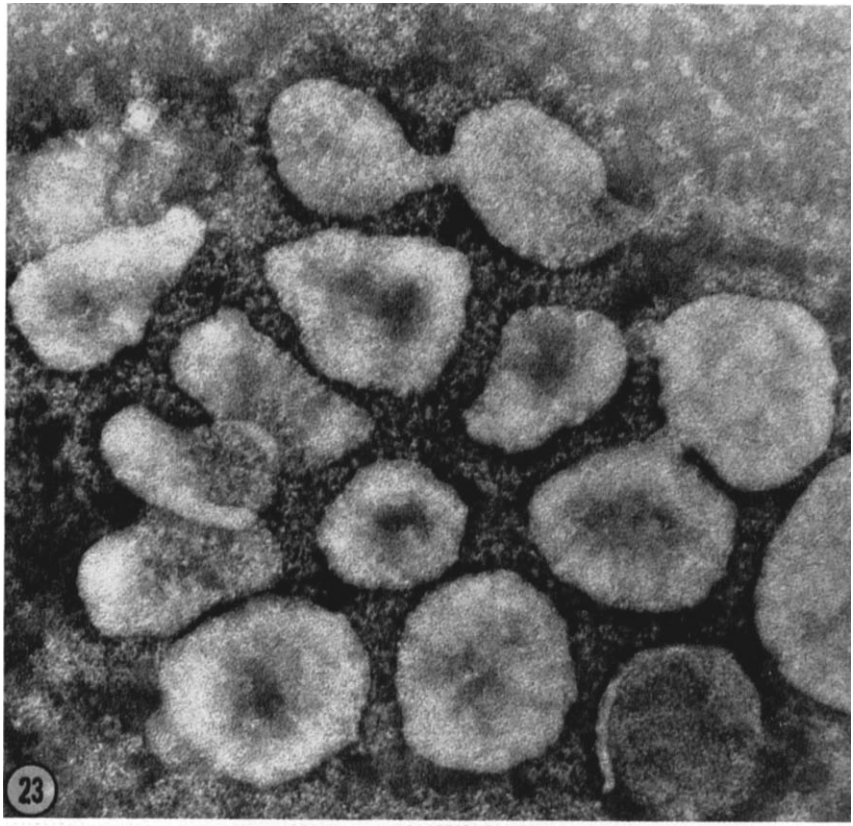
F. Localization of Specific Antigens

The electron microscope counterpart to fluorescent antibody staining is the use of ferritin-conjugated antibody with thin sections, and if it is essential to retain cellular integrity this is almost the only approach possible for the localization of specific antigens. However, since thin sections are normally 200 Å or more thick, the potential of the microscope cannot be fully realized as there is a rule of thumb which states that resolution will never be better than a tenth of the thickness of the object to be examined. In addition, the ferritin marker introduces problems of specificity and is technically not easy to handle. However, if the antigen to be located is on the surface of a structure of viral proportions then negative staining is the technique of choice as it is technically

simple, rapid, and, since the antibody molecule, without a marker, is visualized, questions of specificity do not arise.

To illustrate this aspect of immune electron microscopy we will discuss a group of experiments carried out in our laboratory on avian infectious bronchitis virus. Morphologically the virus is pleomorphic, with distinctive projections (Fig. 22), and is now included in the group known as coronaviruses (Almeida *et al.*, 1968). Complexes were formed of virus and two types of antiserum: (1) homotypic, that is, derived from chicken, and (2) heterotypic, produced in rabbits (Berry and Almeida, 1968). Both of these sera produced clumping (Figs. 23, 25) but at high magnifications obvious differences could be seen between the two types of aggregate. In the case of the homotypic antiserum antibody was attached only to the virus projections and, where these projections were missing, it was clear that no antibody was attached to the envelope of the virus (Figs. 23, 24). The spacing between virus particles was wide and there seemed to be a gap between the antibody-projection complex and the rest of the particle. With the heterotypic antiserum the particles were clumped much more closely together and antibody molecules were attached not only to the projections but also to the envelope of the virus (Fig. 25). A possible explanation of this phenomenon was that the envelope of the virus did not elicit a response in the chicken. The projections, on the other hand, were recognized as foreign and antibody was directed against them. This hypothesis was investigated by reacting the virus with rabbit antiserum produced against normal chick embryo fibroblasts. It was then found that antichick antibody molecules were attached to the envelope of the virus and that virus was neutralized by it. The outstanding feature of the micrographs obtained with this serum is the presence of holes in the virus surface (Fig. 26), a phenomenon that will be discussed in the section on immune lysis.

Other techniques have been able to show the presence of host component in the membrane-bound viruses, but in this instance immune electron microscopy was able to show its presence visually and to localize it. However, it must be admitted that avian infectious bronchitis virus was morphologically an ideal virus for a study such as this, because the projections are very distinct, widely spaced, and frequently missing. This meant that it was possible to localize visually two different species of surface antigen, one of which appears to be antigenically indistinguishable from host cell membrane, the other being virus-specific. Similar studies on viruses such as influenza or the parainfluenza group would probably need virus preparations from which the surface projections could be artificially removed. Such techniques are now becoming available, so that even if the technical approach should be somewhat



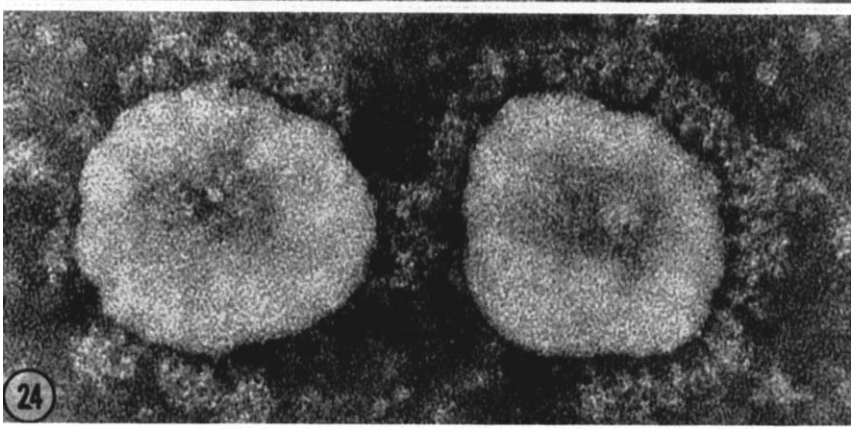


FIG. 23. An aggregate of IB virus and heat-inactivated homotypic (chicken) antiserum. The particles are linked by antibody molecules attached to the projections alone, resulting in a gap between the virus envelope and the projection-antibody complex. This is shown at higher power and more clearly in Fig. 24. Magnification × 200,000.

FIG. 24. A higher power micrograph of the pattern obtained with IB virus and heated homotypic antiserum. The projections are obscured by antibody, but where projections are missing it can be seen that no antibody molecules are attached to the envelope of the virus. Magnification × 300,000.

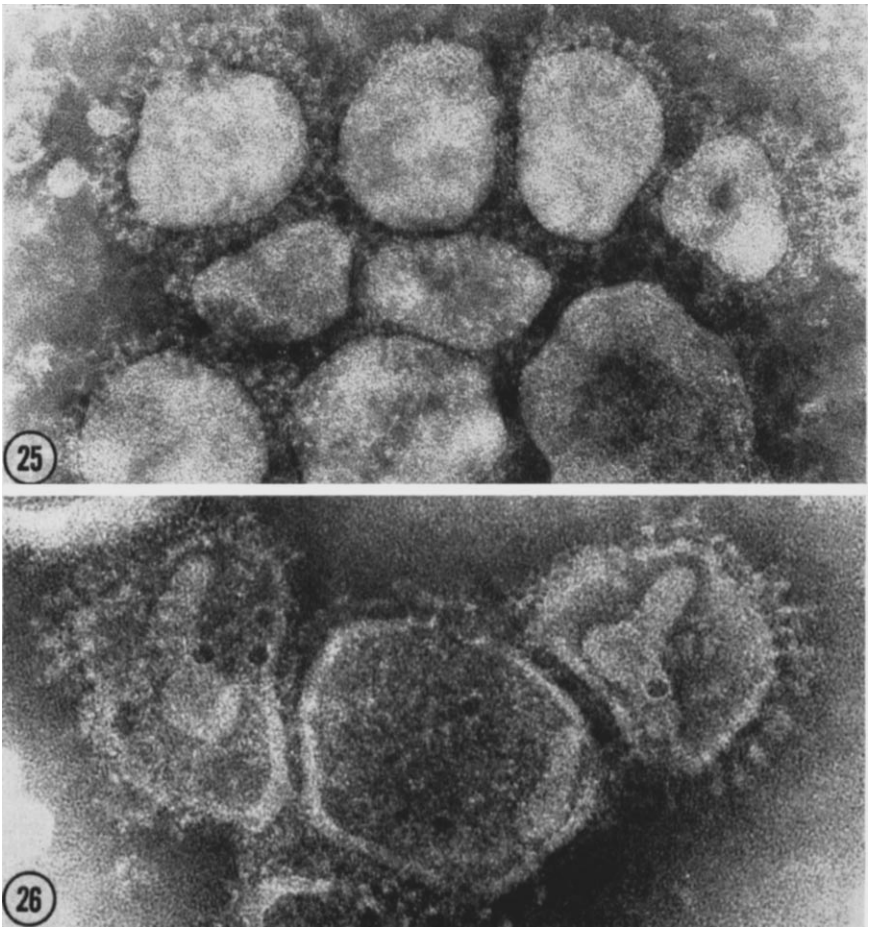


FIG. 25. This micrograph illustrates the effect obtained when IB virus is combined with heated heterotypic (rabbit) antiserum. The particles are closer together than in Fig. 23 and antibody molecules are attached both to the projections and the envelope of the virus. Magnification × 200,000.

FIG. 26. A group of IB particles that have been treated with rabbit antiserum raised against normal chick fibroblasts. Antibody molecules are attached to the viral envelopes and since the serum had not been heat-inactivated lytic holes have appeared in the membrane. Magnification × 300,000.

more complex it still seems possible for a wide range of viruses to be reviewed in this way and specific antigens located. Another problem in this kind of study is that while it might seem possible to use specific antiserum directed against particular virus components, e.g., the neuraminidase of influenza virus, the requirements of the electron microscope

as regards specificity are probably greater than for most other techniques, so that even a small amount of nonspecific antibody will obscure the picture. Again, however, better preparations of antibody are becoming available, and it should soon be possible to utilize the microscope for the double purpose of pinpointing antigens and, when this has been done, for checking the specificity of any particular batch of antiserum.

G. Immune Lysis

The part that complement plays in the immune reaction has long been recognized and put to considerable practical use. In 1964 Borsos *et al.*, using a system of sheep red cell membranes, antibody, and complement, showed that the phenomenon of hemolysis could be visualized in the electron microscope. Negatively stained fragments of red cell membrane exhibited pits or craters approximately 100 Å in diameter. These lesions had a distinct rim surrounding them and it has since been shown that they are associated with damage to the lipoprotein component of the membrane (Humphrey *et al.*, 1967).

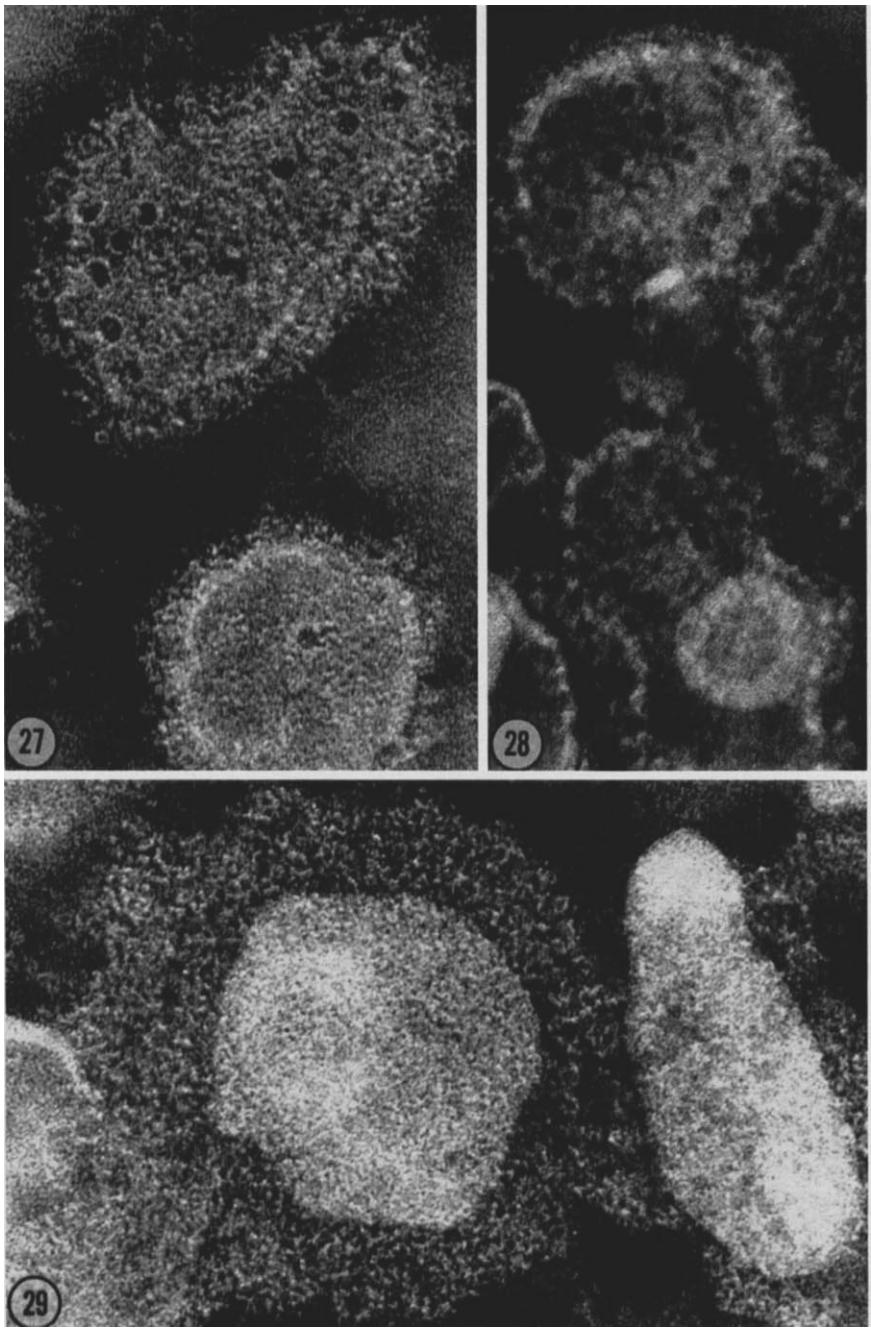
Using the avian infectious bronchitis virus system described earlier (Berry and Almeida, 1968), it has been possible to show that a similar phenomenon, which might be termed virolysis, exists. It had been known for some time that fresh unheated antiserum to infectious bronchitis virus had a neutralizing titer a thousand-fold greater than serum which had been inactivated. This finding held for both homotypic and heterotypic antiserum. In order to investigate this phenomenon, the virus was examined after treatment with each of four groups of antiserum; these were, homotypic fresh, homotypic heat-inactivated, heterotypic fresh, and heterotypic heat-inactivated. As the homotypic serum reacts only with the projections of the virus, changes in the basic envelope could not be expected, as it is known that the enzymic action of complement on lipoprotein can occur only when antibody is attached at or very close to the membrane containing the lipoprotein. Indeed, the only visible difference between the effects of fresh and heated homotypic antiserum was that complement components could be visualized when the unheated serum was used, a finding which will be discussed in the next section. However, the difference in effect between the fresh and heated heterotypic serum was an outstanding one. Those particles treated with heated serum had displayed only the appearance associated with virus-antibody aggregates, i.e., the particles were clumped and the surface detail was obscured by the presence of antibody (Fig. 25). On the other hand, virus complexed with fresh heterotypic antiserum appeared gray, which is indicative of flattening, and displayed pits or craters (Fig. 27) indistinguishable from those present in red cell membranes showing lysis.

The ability to produce virolysis can be restored to heated serum by the addition of guinea pig complement (Fig. 28). Subsequent experiments have shown that the phenomenon also occurs with both influenza and rubella viruses, and although it may be rash to make too general a statement it is conceivable that virolysis is a general occurrence among viruses which incorporate a cell-derived outer membrane. This is perhaps not too unexpected a finding as it is known that cell lipoprotein membranes in general are prone to the lytic action of antibody and complement, and the outer membranes of viruses although virally coded retain many of the properties of the cell membrane from which they are derived.

H. Visualization of Complement

In 1966 Feinstein and Munn showed that a pronounced morphological difference could be seen between bacterial flagella that had been reacted with antibody alone and those that were subsequently treated with complement. The antibody used in these studies was IgM, and the previously described staple forms were seen attached to the flagella. When guinea pig serum was added to this antigen-antibody preparation the appearance changed, as a new complex became visible, attached at first in patches associated with the antibody but eventually merging to cover the complete flagellum. This was bound complement which appeared as a fine fuzzy halo surrounding the antigen-antibody complex. In the avian infectious bronchitis system results similar to this were obtained with both homotypic and heterotypic antisera. With antibody alone the halo of projections plus antibody did not exceed 300 Å, whereas after treatment with unheated serum the halo could extend as far as 700 Å (Fig. 29). However, it should be pointed out that not all particles exhibited this additional covering of fine filamentous molecules. More recently Coombs and Lachmann (1968) have shown that the structure visualized in the electron microscope is C'3, one of the higher molecular weight components of the complement system.

From a virological point of view the results with avian infectious bronchitis using the three-component antigen-antibody-complement system pose an interesting question. Looking only at the heterotypic system it seems feasible to suggest that the enhanced neutralizing ability of fresh antiserum is due to virolysis. One cannot imagine a virus being able to survive as an infective agent after the rupture of the outer membrane. However, fresh homotypic serum has an equally enhanced neutralizing ability (Berry and Almeida, 1968), but no lytic affect can be demonstrated. A possible explanation is that the additional complement coating could act by steric hindrance thus enhancing the effect of a low titer



FIGS. 27–29.

antiserum which by itself could only partially cover a virus with antibody. These findings, that both complement itself and the effects of complement can be visualized, raise the very practical point that antiserum for immune microscopy must be heat-inactivated before use for antibody studies.

IV. Conclusions

In the field of virus study the electron microscope technique of negative staining is now more than 10 years old, and during these years the knowledge of virus fine structure has changed beyond all recognition. The technique has been employed by groups too numerous to mention and the bibliography on the field is now enormous. The extension of the method for immune electron microscopy has been known for 8 years but during this time only a small number of people have attempted to use it. This would seem to be because of two main reasons, first unawareness of the potential of the technique, and second the feeling that the method is an extremely difficult one to handle. By discussing under separate headings the various different aspects that can be studied using immune electron microscopy we hope that we have been able to illustrate its many uses. As for the second point we can only repeat what was stated earlier—that the method is a straightforward one giving direct, visual evidence of the immune reaction to be studied. In the text we have described that the immune aggregate should be incubated for 1 hour at 37°C and then left in the cold overnight. This is the optimum approach for a system where the concentration of neither the antigen nor the antiserum is known and maximum clumping is needed. However, with many systems these parameters have been established, and if it is known that clumping can be obtained readily, for example as with the avian infectious bronchitis system, then incubation for 1 hour at 37°C is sufficient, followed by spinning at 10,000 rpm for half an hour. This means that the whole preparatory procedure can be carried out in less

Fig. 27. When nonheat-inactivated heterotypic antiserum is combined with IB virus then the three component antigen-antibody-complement system leads not only to attachment of antibody but also to the appearance of lytic holes in the virus envelope. These holes are approximately 100 Å in diameter and resemble those that occur in red cell membranes during hemolysis. Magnification × 300,000.

Fig. 28. The ability to produce lytic holes can be restored to heated heterotypic antiserum by the addition of either fresh normal serum or guinea pig complement. This micrograph was produced by using heated rabbit antiserum plus fresh normal rabbit serum. Magnification × 300,000.

Fig. 29. IB virus combined with nonheat-inactivated homotypic antiserum allows the visualization not only of antibody molecules but also of certain components of complement. With antibody alone the halo surrounding the virus is approximately 250 Å deep while here it is in the 700 Å range. Magnification × 300,000.

than 2 hours, a time comparing favorably with most other immunological techniques. Also in favor of the technique is the fact that it can be carried out with very small quantities of material. With some problems the absolute amount of virus available for any one experiment has been in the region of 10^6 particles, an amount that would be too small to give a result with the agar gel immunodiffusion techniques or indeed with most standard immunological techniques. The limitations of the method are mostly concerned with the type of structure on which the antigenic sites are located, size being all important. Viruses in the small to intermediate size range are ideal for immune studies, as the particles are not disproportionately larger than the antibody molecules attached to them, and interaction between them can be visualized with better than 10 Å resolution. Particles as large as the poxviruses are becoming too large for good resolution of antibody molecules to be obtained, and aggregates of poxvirus particles are usually too dense to be recorded photographically except at low power. If, on the other hand, the method is needed only to establish whether or not aggregation has occurred then structures considerably larger than the pox group, e.g., mycoplasmas, can be employed.

Having considered the results obtained with the technique of immune electron microscopy and taking into account the limitations and advantages of the method it is worthwhile asking what possible future uses it may have. In the field of clinical virology there is a possibility that the electron microscope technique of negative staining will become a standard method of diagnosis (Williams *et al.*, 1962). Already the method has proved its value for rapid diagnosis in smallpox outbreaks (Peters *et al.*, 1962; Cruickshank *et al.*, 1966). It is not hard to imagine that an extension of this approach, employing immune electron microscopy, will allow particles not only to be visualized but also to be serotyped. In a few instances this could be done directly from the patient when, for example, vesicular lesions containing relatively large amounts of virus are present. If a herpes-type virus were found it would be possible by the use of typing sera to establish whether it is herpes simplex or herpes zoster. This finding at the moment may be of academic importance only, but a time should come when specific therapy is available, and it would then be of the utmost importance to serotype such viruses in as short a time as possible. Also in the routine field immune electron microscopy could be both the simplest and the most accurate method for checking the specificity of an antiserum and even of titrating it, once further base lines have been established.

As far as research is concerned the present article seems to have described the beginnings that have been made in the visualization of several aspects of antigen-antibody interaction. Each line investigated has

yielded a few answers but, of much greater importance, has led to a better understanding of what questions should be asked. At the moment viral antigens can only occasionally be obtained in pure form so that there are only a very few systems where controlled and specific virus-antigen-antibody systems can be visualized. The situation will almost certainly be altered in the next few years, and it will then be possible to build up a picture of virus particles not only from the morphological but also from the antigenic viewpoint.

To conclude, we cannot do better than to reiterate the sentiments put forward 30 years ago by Anderson and Stanley (1941): "The present results demonstrate the usefulness of the asymmetrically-shaped tobacco mosaic virus and the advisability of making extensive studies of the antigen-antibody reaction by means of the electron microscope" (p. 343), perhaps adding one small amendment; for the phrase "asymmetrically-shaped tobacco mosaic virus" one could now substitute "the distinctive appearance of negatively stained virus particles."

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