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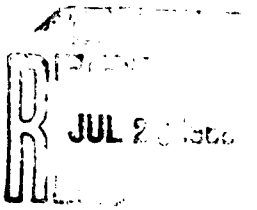

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The morphology of viruses pathogenic for man and animals.

by Dietrich Peters.

Translated from: Zentralblatt fuer Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, I Orig. 176: 259-294 (1959), by the Technical Library, Technical Information Division.

Since the comprehensive coverage of electron-microscopic findings in the field of virus research was published by H. Ruska in 1950 (1), the rapid development of this domain has resulted in considerable changes in approach.. While in earlier years most studies were concentrated on the size and shape of isolated virus particles, current investigations deal with the various phases of the system host cell-virus, especially the processes during infection, during viral propagation within the cell and, not to a lesser extent, with the detailed structures of the virus particles proper, their biochemical composition and the functional significance of their subordinate factors.

The expansion of subject matter for research resulted by necessity from the increase in methodical approaches occurring primarily in the field of electron-optical techniques of preparation. In addition, the extension of light-optical methods, brought about by such advances as the introduction of fluorescent antibodies (2), enriched the science of virology with valuable new insights (3). The most decisive progress, however, was doubtless offered by the method of ultracytology, which in recent years has been developed to a high degree of perfection (4). Since it became possible to obtain sections from infected tissue 30-50 millimicrons thick, and to study these electron-microscopically, this method has opened to virology a field of application of inestimable value, a field that is characterized by bridging the gap between morphology and macromolecular chemistry.

The present compilation is based on a number of selected, typical examples in an ascending order of magnitude, permitting facile demonstration of the advances made during the past years. The greater emphasis placed on the group of poxviruses is justified by the author's particularly intensive study of these viruses. Besides, morphological knowledge, which ought to receive principal consideration in this paper, has seen particularly great advances among this group. A number of recent compilations have covered the entire field in a less accentuated fashion (5, 6, 7).

The expression "elementary body" will be employed repeatedly henceforth. The term "elementary body" was coined during the light-optical era of virus research and has since been used almost exclusively for the description of those types of virus that are just visible with the light microscope, i.e. essentially the viruses of the psittacosis-lymphogranuloma inguinale group with a diameter of 400-500 mμ and those of the pox group, 250 mμ in diameter. Since electron-optical methods have now revealed numerous other viruses in the form of infectious particles, it seems quite proper to extend this term to virus particles of smaller dimension, e.g. to those of foot and mouth disease, which were recently confirmed electron-optically and which measure 22 mμ, i.e. smaller by one decimal power (8). Moreover, the diminutive form customary in the German literature should be abolished in view of the shift in size. The term "elementary body" will therefore be synonymous with "virus particle" in the following elucidations.

Poliomyelitis virus.

The modern electron microscope's power of resolution under optimal conditions of contrast is currently near 1 mμ (9), with optically less favorable depiction of ultra-thin tissue sections, about 3-5 mμ (10). Consequently there are no physical obstacles to the identification of the smallest currently known elementary bodies of about 20 mμ within the infected cell. However, the difficulty of identifying the particles as viral bodies and differentiating them from the native, detailed structure of the normal cell or, even more so, of the pathologically changed cell, is compounded with decreasing particle size. Thus, for example, the rarely publicized attempts at intracellular localization of poliovirus which, with a diameter of 27 mμ, belongs to the smallest currently known viruses, have so far given no distinct clues to a positive identification of elementary bodies within the host cell (11, 12, 12a); they merely show the difficulties associated with studies of small viruses.

On the other hand, the gain in knowledge of these viruses is quite considerable, thanks to the application of conventional methods of electron microscopy. The most important prerequisite for the morphological study of small viruses, the production of highly purified suspensions, was met a few years ago by the feasibility of inducing viral propagation in tissue cultures and by methodical perfection of enrichment and purification. Treatment of very large quantities of infected cell cultures yielded poliovirus (13, 4; summation 15) in 1955 and the related Coxsackie virus (16) in 1956 in a degree of purification so high that crystallization was induced. Thus, proof was offered twenty years after the first report of successful crystallization of a phytopathogenic virus, that there are crystallizable viruses among those pathogenic for animals. Poliovirus further resembles plant viruses, e.g. bushy stunt virus of the tomato, in view of its content of about 25% RNA, an abnormally high level for animal viruses.

Electron-optical studies carried out on such crystals by means of the replica technique show that elementary bodies of probably spherical structure are combined isometrically to a crystal lattice without matrix. The particle size within the crystal amounts to 27.3 ± 1.4 mμ (17) in the case of poliovirus, and 28 mμ (16) in the case of Coxsackie virus. From the viewpoint of morphology, the fact that 90-99% of the elementary bodies included in the crystal lattice turn out to be inactive upon quantitative evaluation, may appear to be a mere aesthetical shortcoming; this circumstance is probably induced by prolonged treatment. The crystallizability of a virus is a remarkable factor and certainly represents an expression of extreme uniformity among the individual virus particles. This does not clarify the internal arrangement of the elementary bodies, however. Even though the chemist will be tempted to presuppose macromolecules in the crystal lattice, the morphologist reserves the right to consider the basic units, by his criteria, as structurally indivisible corpuscles. Further investigations of the fine structure of these elementary bodies are indispensable. At any rate, it is worth mentioning in this connection that a differentiation between an outer membrane and an internal body, designated hypothetically as "nucleoid," has already been described (18). Illustrations by other authors (e.g. 19) also point to a sub-structure of the particles, including electron micrographs of sectioned virus sediments (20) and evaluations of X-ray diagrams (21), which suggest that the centrally located RNA is surrounded by a layer consisting of 60 protein subunits.

Adenovirus.

As may be expected, the conditions are more favorable in the clarification of larger elementary bodies. When the particles are doubled in size, i.e. to about 60 mμ, currently available methods have been found adequate for the differentiation of elementary bodies and cellular structures, since all virus particles studied to date have revealed characteristic internal structures. As in the case of light-optical demonstration of relatively large bodies, e.g. rickettsia, it is difficult to identify viruses with certainty if their concentration in the cell is small. Compared to light-optics, this difficulty is compounded by three very important factors: The insubstantial thickness of the sections (one section encompasses only about 1/500th of the cell volume), limitation of the visible cellular surface imposed by magnification, and the absence of color hues. The conditions are particularly favorable when the elementary bodies are easily recognized by an aggregate arrangement. A remarkable example of this state is offered by the adenovirus, a group of pathogens of the respiratory tract discovered only a few years ago (22, 23) which have been subjected to intense study in various quarters in view of their morphological attractions. When an HeLa cell culture is sectioned 24 hours after infection or later, the interior of the cell nuclei quite regularly reveal elementary body aggregates which extend across several microns and which therefore can be identified light-optically (24).

The most important findings, confirmed by other authors, shall be demonstrated below by means of a few illustrations of adenovirus, Type 3, obtained in collaboration with K. H. Andres and G. Nielsen. Fig. 1 shows a relatively early stage of infection in a barely affected HeLa cell. In addition to numerous aggregations of elementary bodies, the cell nucleus contains several centers of density with variable contrast. Current interpretations establish a close relationship of these centers with the formation of elementary bodies, without the benefit of strict proof. Closer scrutiny leads to the surprising discovery that the majority of viral aggregations are delineated by a polygonal contour and that the elementary bodies in the interior of the aggregates form a regular crystal lattice (Fig. 2) (25-29). Thus, a condition that could be shown in the case of poliovirus only with purified viral suspensions in vitro, already manifests itself persuasively in vivo when dealing with adenovirus. The tendency to crystal formation is variable among the different types of adenovirus. When the structure of the cell nucleus is destroyed in the onward course of infection, the crystals pass into the cytoplasm and are gradually dissolved therein, ultimately resulting in a free dispersion of elementary bodies in the plasma. As depicted in Fig. 3, regular observation is made in this connection of concentrically oriented membranous systems -- evidently constructed by the cell -- which subject the virus particles to areal isolation from the remaining plasma (26).

In contrast to several other viruses, only one basic form of elementary body is currently known. The particles in the cytoplasm cannot be differentiated from those in the cell nucleus. They have a diameter of about 60-65 $m\mu$. Electron-optical information about their interior structure is therefore obtained with greater certainty than in the case of poliovirus. Several authors agree in describing an inner body (probably containing DNA), a peripheral, lighter zone and a surrounding membrane (Fig. 4). It is true that the inner body may be absent in isolated cases (25, 29). Such elementary bodies possibly represent particles that are not yet or no longer infectious. They may correspond to those elementary bodies from purified suspensions that deviate from normal owing to a translucent center (30). There is less agreement on the external shape of the particles. The aforementioned authors considered them to be spherical, based on sections fixed with osmium tetroxide. As Andres and Nielsen (31) were able to show in our research group, a different picture is produced when fixation is accomplished with potassium permanganate. This type of fixation was most successful after "plexigum" had been found to be a suitable imbedding agent (32). This process produces distinct polygonal delineation of elementary bodies, not only when deposited within a crystal (Fig. 4a), where this could be caused by dense packing, but also after its loosening or dissolution in the cytoplasm (Fig. 4b). It is possible that fixation with OsO_4 fails to adequately contrast the virus particles' periphery, thus being unable to visualize the outermost layer. This result agrees with the experience of other authors (33, 34)

who also suspected a polyhedral shape, based on freely prepared elementary bodies. Icosahedra and rhombic dodecahedra have been considered. Based on personal experience gained from preparations fixed with $KMnO_4$, we prefer the latter in the case of adenovirus (Type 3) (31).

To my knowledge, purified particles have not been subjected to biochemical analyses to date. It was established in elegant fashion by means of sections of variable thickness, that the crystalline aggregates will normally produce positive Feulgen reactions. They differ clearly from nucleic chromatin by refusing to absorb the pigment upon overstaining with azure B (35). Since no special matrix was found electron-optically within the crystalline aggregates, the content of DNA was ascribed to the elementary bodies proper.

Particular attention is due to the fact (at least, as far as Type 5 is concerned) that Feulgen-negative intranucleic protein crystals up to 20 μ in length were observed in addition to those just described, i.e. structures that contain very little or no DNA (36, 37). Since such bodies were found predominantly in early stadia of infection, it is not improbable that these are aggregates of preliminary stages of intact elementary bodies, possibly virus protein that is not yet equipped with virus-specific DNA. Other manifestations, such as the uniform distribution of isolated elementary bodies over large nucleic areas or the appearance of Feulgen-positive aggregates in the cytoplasm of the infected cell, could be interpreted as being an expression of a developmental cycle of the virus, linked to the host cell's degeneration.

Herpes viruses.

Viruses appearing in the nucleus include the virus of herpes simplex, again twice as large as the preceding pathogen. In free preparations, it shows, as do the adenoviruses, transitions containing both spherical particles with dense centers and those that have relatively translucent centers (38, 39). Detailed results were obtained from thin sections of cells infected with herpes simplex (40, 41). About 12 hours after infection, the first morphologically secured sign of viral propagation appears in the cell nucleus in the form of small, dense particles about 40 mμ in diameter. These primary bodies subsequently reach a dimension of 70-100 mμ by the addition of an enveloping membrane, and later, during their exit into the cytoplasm, acquire a second membrane and a total size of 120-130 mμ. In this case, three different phases of viral development are thus clearly delineated. It is quite probable that the terminal stage marked by a dual membrane represents the mature, fully infective virus, as it is encountered in extracellular space. Similar conditions were observed in the case of herpes B virus (42). Parallel to the results obtained from adenovirus, a certain strain of herpes simplex virus (43) and herpes B virus (44) have also been described as possessing a crystalline arrangement of intranucleic elementary bodies, i.e. those having only one membrane. The tendency

of virus particles to incorporate themselves in a crystal lattice apparently is weaker among the herpes group than among adenoviruses. The most important prerequisite of such an arrangement, i.e. the uniformity of individual particles, probably is not as ideally pronounced among the more labile herpes viruses.

There is some support for the assumption that the herpes elementary body receives its second membrane during its transfer from the nucleus to the cytoplasm and that this envelope is materially contributed by a normal component of the host cell, perhaps the nuclear membrane. During this process of envelopment, it may happen that two elementary bodies are occasionally surrounded by one outer membrane, as has been observed in isolated cases in connection with herpes B virus (42). The term "membrane," carried over from the literature, does not indicate possible functional performances of the corresponding structure in this connection. The same circumstance could be more correctly clarified with the concept of"layer". Since the mature elementary bodies contain a zone of weaker contrast between the inner "membrane" and the newly acquired one, it is quite probable that this layer is also attached to the virus particles at a later time.

Virus of mouse mammary gland cancer.

The process of envelopment of virus-specific particles by a sheath preformed by the host cell seems to involve a generalized principle. Comparative observations have been made in connection with other viruses, e.g. the virus of mouse mammary gland cancer (45), whose development was recently studied electron-optically in tissue explants (46, 47). In this case, the barrier at which envelopment takes place is not the nuclear membrane, but apparently the cell wall. The well-defined, intracellular elementary bodies which had been preformed in the cytoplasm, leave the cell by way of protrusions in the cell wall (microvilli) and probably acquire cell wall material in the process.

Myxoviruses.

Among myxoviruses, the conditions of multiplication are similar and yet, in many respects, different. (This group has recently been treated in this journal by a representative discussion of the virus of classical fowl cholera (48, cf. also 49-51). It will be considered only briefly within the present framework, compared to the extensive literature.) These viruses also receive their ultimate structure only at the cell wall. However, an interesting and very important difference vis-a-vis the viruses discussed heretofore, consists of the circumstance that electron-optically identifiable preliminary stages of mature elementary bodies have been found neither in the cell nucleus nor in the host cell's cytoplasm. Definable, complete virus particles of this group apparently exist only outside the cell. It is known from serological and fluorescence-optical studies of influenza virus and the virus of classical fowl cholera, that virus-specific preliminary stages --- a complement-fixing, RNA-containing antigen and an hemagglutinin --- are demonstrable in the host cell at an early stadium. A positive electron-

optical proof of these subunits within the cell has not been secured to date, however. Aside from lipid, these two factors are reencountered in the intact, infectious elementary body after the termination of synthesis. When the virus particle is decomposed in ether, both are liberated anew and electron-optically measurable antigen particles 12-17 mμ in size are obtained, part of which show oblong aggregation, together with spherical particles of hemagglutinin about 30 mμ in diameter. Based on similar results as well as serological and chemical findings, models of elementary bodies have been constructed for the viruses of classical fowl cholera (48) and influenza (52) which, in addition to various differences, share a central arrangement of RNA-containing antigen and a peripheral location of hemagglutinin.

A differentiation of the internal structure of intact elementary bodies of the myxovirus group, shown in toto, has not succeeded to date, although this has been accomplished with sectioned particles (53, 54, cf. also 55). Preparations fixed with OsO_4 have revealed a dense inner body about 20 mμ in size, surrounded by a lighter zone, a sharply demarcated membrane and, finally, by a more diffuse, external envelope of lower density. The diameter of the unimpaired particle is 70-80 mμ. If fixation is done with $KMnO_4$ instead of with OsO_4, as utilized by M. E. Bayer in his study of hemagglutination by influenza virus A/Singapore (56), an insight of comparable quality is gained into the particle's internal structure (Fig. 5). However, material subjected to this type of fixation and optimal section seems to reveal inner bodies with a light center; moreover, the peripheral enveloping substance appears as the most strongly contrasted structure. The contrasting of detailed structures probably depends in large measure on the type of fixation. Judging by currently available electron micrographs, the various components of these elementary bodies consist of concentrically arranged layers. So far there has been no electron-optical support for an arrangement of hemagglutinin in the form of 6 individual particles 30 mμ in size, as contained in the model of the classical fowl cholera virus (48). Special attention is due also to Bayer's observation (56) that fixation with $KMnO_4$ promotes the visualization of a polygonal delineation of elementary bodies of influenza virus, analogous to the findings relative to adenovirus (see above) (Fig. 5a). Future investigations will establish whether or not an artificial phenomenon is involved.

In addition to the discussed infectious virus particles, generally referred to as spherical, there have been known for some time filamentous forms possessing low infectivity or none at all. Sectional preparations reveal the absence of an inner structure corresponding to the central core; such an inclusion may become apparent only toward the end, where they occasionally assume a structure resembling an elementary body. The so-called incomplete viruses also appear to be hollow in sections (54); they represent elementary bodies capable of hemagglutination (Fig. 6), but not of infectivity. Their dimensions, between 50 and

120 mμ in diameter, fluctuate more widely than those of complete virus. There is considerable support for the assumption that the virus particle's capability to induce infection is intimately tied to the existence of an inner body. This would be eminently clear if the latter contained RNA, as suggested by the results mentioned above. The link between serologic and chemical findings on one hand, and morphological results on the other, has not been established unequivocally in the case of myxoviruses.

Poxviruses.

In this respect, knowledge of the viruses in the pox group has seen considerably greater progress, in spite of their rather complicated structure. Their stability, size and, especially, their typical shape proved to be extremely favorable for morphological investigation. As recognized at an early date, the most striking structural characteristic of this group is the "brick shape." It permitted the consolidation of the viruses of variola vera, vaccinia, molluscum contagiosum, fowl and canary pox, ectromelia and rabbit myxoma in the group of socalled brick viruses (57). This classification is immediately supported by biological similarity; it is ultimately justified on the basis of additional morphological findings. Nowadays, this group includes the virus of rabbit fibroma (Shope) besides the pathogens of other animal pox diseases; perhaps it will be increased by the addition of still other viruses, e.g. those of stomatitis pulposa and contagious pustular dermatitis. No positive virus-specific differences have been discovered within this group, either in the structure of elementary bodies or in morphologically visible processes of their genesis, other than certain variations in their external form. Pronounced deviations are known only from the later stage of infection, the phase of inclusion body formation. The virus of vaccinia, studied most intensely to date, will serve as a model in the description of elementary bodies proper and of the phenomena preceding their development.

The morphology of mature vaccinia elementary bodies.

Suspensions of mature elementary bodies are obtained without particular difficulty by homogenization of infected rabbit skin and differential centrifugation. However, direct preparations of fully infected tissue, e.g. rabbit cornea, chorioallantoic membrane, HeLa cell cultures, generally lead to practicable results (58, 59). The lesser purity of such preparations is compensated by the advantage that the factor of selection, unavoidable in the course of repeated centrifugation, is absent. Regardless of the type of preparation, mature elementary bodies regularly present a brick shape with rounded corners (Fig. 7 and 8). The typical form considerably facilitates differentiation between virus particles and cellular debris, and thereby offers favorable conditions for rapid electron-optical confirmation of variola diagnosis (60). Dimensional data fluctuate considerably for the various

viruses of the pox group; their length, for instance, ranges from 230 to 320 mμ . No inferences of confirmed deviation in size should be made from this data, however, since the results imparted so far are still marked by various imperfections. The situation is similar with respect to the axial ratio length:width:height, which ought to be fairly established at an average of 2:1.5:1 for all members of the pox group.

As long as more suitable electron-optical methods were non-existent, there was no shortage of attempts to demonstrate, by means of measurements, that virus particles do or do not multiply by division (61, 62, 58). The concensus was that no positive dividing forms had ever been observed and that the variation in size shown by elementary bodies did not exclude division, but that this variation was still considerably smaller than in the case of cocci in their fissional phase, for example. Mature elementary bodies therefore are best interpreted as an expression of a latent stage, comparable to microcysts or spores (63).

In customary preparations, the elementary bodies appear almost invariably in a horizontal position. However, when the interfacial forces are avoided or attenuated during drying, as rendered feasible by means of the socalled "critical point" method (64) or by preparations with isopentane (65), numerous bodies are regularly seen in a vertical position (Fig. 7, 8 and 12 c-d) (66). The third dimension becomes directly visible thereby. Preparations of this type already show that elementary bodies are not constructed homogeneously. Horizontally positioned particles invariably reveal a central density; contrasting aggregates of lesser degree are frequently found also in the four corners (Fig. 7 and 12a) (67). In preparations treated with vapor, the central density is particularly emphasized by a round depression (Fig. 8 and 12b). The profile of elementary bodies is characterized by a bilateral protrusion (Fig. 7, 8 and 12 c-d). In spite of greater contrast, the vertically placed particle reveals, along the long axis, a distinct delineation of a strongly contrasted dumbbell-shaped internal structure from an external layer of lesser density (Fig. 7).

A process connected with electron irradiation, whose detailed mechanism is not yet clear, permits a very specific illumination of this particular zone (Fig. 9) (68). As preliminary steps, the elementary bodies are first dehydrated and treated with such unpolar solvents as carbon tetrachloride or heptane. The horizontal elementary bodies indicate that this process apparently begins in the four corners and spreads circularly around the central density. This phenomenon could be dismissed as an undesired artifact if it were not marked by high specificity and did not affect that special internal structure which, as will be discussed later, has been recognized as the vehicle of DNA.

Structural analysis by means of fermentive degradation.

Greater insight into the anatomy of elementary bodies is obtained by gradual degradation, i.e. by aimed isolation of individual detailed structures. However, methods of dissolution are successful only when marked by a high specificity. Alkali, for example, is unsuitable, since its action is not specific (67). This condition is met ideally, upon proper application, by highly purified proteolytic or nucleic acid-splitting enzymes. This approach became feasible after Dawson and McFarlane (69) had shown that the peripheral layer of the vaccinia elementary body is lysed by pepsin; a sharply delineated core remained within a resistant enveloping membrane (Fig. 10). DNA, found to be present in a concentration of 5.6% (70), remained undissolved during this process, justifying the assumption of its localization in the inner body. The ultimate proof of this circumstance was finally delivered by the application of a method which had been perfected previously in work with bacterial nuclei (71, 72; cf. also 73). The inner body, isolated with pepsin, was brought into specific solution with DNase and a second treatment with pepsin in the case of elementary bodies of vaccinia (74), molluscum contagiosum (75, 63) and canary pox (76); the result was partly or completely empty membranes, as depicted in Fig. 12 u-x. Depolymerization of DNA by nuclease cannot be observed for the time being, since the mass density of the core is not essentially decreased thereby. The fact that the inner body, being pepsin-stable at the start, is rendered pepsin-sensitive after treatment with nuclease, shows unequivocally that the enzyme had been effective. It is understood that pepsin can be replaced by papain at pH 4.2 in both phases of this reaction (77). The inner body therefore consists of a desoxyribonucleoproteide. A considerable portion of the protein component may also be lysed with weak hydrochloric acid after treatment with DNase; it probably contains a protein of the histone type (74). The occurrence of RNA in vaccinia elementary bodies has not been excluded or confirmed biochemically to date. Several tests have failed to produce a morphologically demonstrable substrate for ribonuclease.

The DNA-containing inner body is not a compact structure; suitable fixation reveals it in the form of an oblong disc that is lent an annular aspect by a central depression and often presents the profile of a short, dumbbell-shaped rod (Fig. 12 i-l). Apparently it is not identical with the socalled "central density" (78; cf. also 63, 76). Frequently the contrast of the core to the longitudinal axis of the elementary body is strongly accented, giving the impression of a dual structure (Fig. 13b).

The "central density" is another structural element that becomes visible in the center of the core in level elementary bodies when the action of pepsin is short-lived (78) or at less favorable pH values (e.g. 3.5 instead of 1.5) (Fig. 12 e-f) (79). More favorable conditions

for its observation are created when the annular core is removed in addition to the peripheral layer, e.g. during hydrolysis with papain (77) or trypsin (80) in the neutral range. In level elementary bodies, this element thereupon appears in the expected central location (Fig. 12 q-r). In originally vertically positioned particles, on the other hand, two laterally arranged bodies become visible under these conditions (Fig. 12 s-t, 13 c-d). For this reason, the socalled "central density" of horizontal elementary bodies apparently represents the combined contrast of two superimposed bodies.

It follows from the preceding results that mature elementary bodies consist of at least four substructures: 1. an enveloping membrane, 2. a peripheral protein-containing layer, 3. a DNA-containing, discal or annular inner body and 4. a dual structural element corresponding to the socalled "central density."

As in light-optics, electron-optical results also depend on the correct choice of fixatives. Our objects yielded excellent results upon fixation with alcohol-acetic acid, although OsO_4, preferred in electron microscopy, was utilized with fairly good success when the enzymatic reaction was subsequently carried out in a reducing milieu (79). Systematic studies (66) with pepsin (79), papain (77) and trypsin (80) of the relation between enzymatic reaction and pH or the ionic milieu upon a given fixation ultimately led to the recognition of structure-specific limits of reaction, considered to be characteristics of the substructures. Papain and trypsin reveal a morphologically demonstrable effect only when a fixation (denaturation) of elementary bodies has preceded. In the case of pepsin, denaturation is automatic through the agency of the acid medium. Fig. 11 gives a compilation of resulting data, obtained after fixation with alcohol-acetic acid. Since the enveloping membrane normally is resistant to protease, similarly to bacterial cell walls, it was not included in the graph. Judging by its reaction to the three enzymes, the peripheral layer consists of protein. The DNA-containing core is resistant to pepsin at all levels of pH. It is digested by papain only at values above pH 4.8, although the enzyme may still be effective proteolytically in the acid reaction, as shown by the degradation of the peripheral zone. Apparently the charge of the substrate is changed at the transition point pH 4.8, followed by a shift in the stability of papain. Parallel to the behavior of cell nuclei (81, 71, 72), the inner body is stable against dialysed trypsin, but labile against the same ferment in the presence of ions. In this circumstance it again reveals the typical behavior of desoxyribonucleoproteides. The dual structure designated as "central density" occupies an intermediate position with respect to its composition. In most elementary bodies it is digested under favorable conditions of pH, just as the peripheral layer. On the other hand, it consistently proves stable against all three enzymes in a few particles in all transitions. This indicates that the composition of this substructure varies in the elementary bodies. It is very probable that a protein is involved here; however, it is contained in some particles in a protected form.

It follows from Fig. 11 that a gradual degradation of elementary bodies is possible, depending on the choice of enzyme and pH. Fig. 12 gives the results by means of typical examples of micrographs of horizontally and vertically positioned particles in unshadowed and shadowed preparations. When the peripheral layer is digested alone (e.g. pepsin in 0.1 mole of cysteine, pH 4.0), the annular core and the socalled "central density" become visible within the membrane (Fig. 12 e-h). When the latter is dissolved (e.g. pepsin pH 2.0), the core alone is retained in the membrane, its annular form being confirmed by the lateral aspect (Fig. 12 i-l). If the same process is carried out with papain at pH 4.2, the membrane itself is frequently digested under conditions that are not yet clear; as a result, inner bodies are isolated whose additional structural details are distinctly discernible in the form of a square shape with densities in the corner zones (Fig. 12 m-p). As already indicated, the dual element ("central density") can also be demonstrated within the membrane by simultaneous lysis of the peripheral layer and the core (papain pH $>$ 5.0 or, preferably, ion-containing trypsin, pH $>$ 6.0) (Fig. 12 q-t). In addition, empty membranes are obtained, usually in great preponderance, depending on the conditions of reaction (Fig. 12 u-x), as produced also by the combination pepsin - DNase - pepsin (see above).

The described three-dimensional structure of elementary bodies recently found additional confirmation. When the virus particles are exposed to a very low charge density, thereby dispensing with precise focus, it is possible subsequently to carry out a successful enzymatic digestion. We succeeded in observing the fate of individual elementary bodies in minute detail (82). For example, a comparison of Fig. 13a and b shows that an elementary body which originally was positioned vertically (V) reveals the core in profile after treatment with pepsin. (The elementary body labeled V' originally presented a side view, then assumed a horizontal position upon exposure to enzymatic action). Even more impressive is the proof of the structural arrangement of the dual element, as revealed in Fig. 13 c and d. While papain (pH 7.0) exposes only one central body in level virus particles, both laterally positioned elements become distinctly visible in three vertical elementary bodies (V), and only in those.

Structural analysis by means of ultramicrotomy.

The use of ultramicrotomy eventually led to complete confirmation of biochemical-morphological findings. Although studies of infected tissues had produced characteristic signs of the sectioned mature elementary body (83, 84), the picture was complemented by systematic investigation of suspended elementary bodies (66, 85). The basic structure therefore is independent of the fixative in its essential factors (Fig. 14) and may thus be considered to be definitive. It is true that the contrast is poorer upon use of organic fixing agents

(e.g. alcohol-acetic acid and formaldehyde), when compared to fixation with OsO_4 (Fig. 14 a-b) or $KMnO_4$. This disadvantage is easily cancelled, however, by contrasting the prepared sections with uranylic salts (Fig. 14 c-h). Fig. 15 depicts current knowledge schematically. The brick-shaped form of the elementary bodies indicates the existence of two distinct sectional planes, a horizontal and a vertical one (cf. also Fig. 20c). Based on experience, ideal horizontal sections are more rarely observed than vertical ones; this may be expected, judging from the schematic representation.

According to the laws of electron optics, sections possessing a thickness of 30 mμ under favorable conditions yield a better resolution than the elementary bodies proper (120-140 mμ) or the core surrounded by the limiting membrane. For this reason, higher required magnifications are possible and additional structural details become visible. The basic components are identical, however, as already described by means of biochemical degradation.

The enveloping membrane normally appears with dual contours; fixation with alcohol-acetic acid produces a single line. The peripheral protein layer has a fine, homogeneous structure. It borders directly on the outer membrane in the peripheral zones of the elementary body. However, vertical sections show that the dual element (identical with the "central density") is imbedded in the median portion between both structures. This element is always more strongly contrasted than the peripheral layer of protein. The dual element is not found in ideal horizontal sections. In the center, we finally see the discal, biconcave core, which deserves special attention due to its content of DNA. Its limiting peripheral zone usually shows a strong contrast, which is particularly vivid following treatment with uranylic salts which apparently have an affinity for this substructure. Suitable fixation again produces a dual structure (Fig. 14 e-h); the contrast of the two contours is quantitatively variable, however. Following fixation with formaldehyde (and, usually, with OsO_4) the core shows a strong contrast even in its interior portion. Fixation with $KMnO_4$ leads to contrasts in the core and its dual contour which are not immediately reconcilable with the results presented here (Fig. 20 b-c) (86). The core doubtless is the most sensitive structure of the elementary body; this was already indicated by the specific loss of contrast owing to electronic factors (see above). Serious consideration must be given to artifacts. However, since this structure undoubtedly represents the most interesting subunit of the elementary body, its subjection to detailed analysis ought to prove fruitful.

Initial signs of detailed structures of this element are found already upon closer observation. Thus, for example, Fig. 14b shows a granule in the upper left area of the inner body, surrounded by a lighter zone; the cut through the other three corners produced only the light zone (cf. also the second drawing in Fig. 15). Such forms are also

found in vertical sections, if these have been made diagonally through the elementary body, as expressed by the lower drawing in Fig. 15. These forms very probably are identical with the four zones of density found in the intact particle (Fig. 12a), with the zones in which specific contrast loss commences (Fig. 9) and, finally, with the four corner zones of the exposed core (Fig. 12m).

Based on the results described, the anatomy of the mature elementary body turns out to be highly differentiated. The discovery of the structure has thereby outdistanced knowledge of the substructural functions; future studies must endeavor to close this gap.

Stages of the elementary body.

It would seem indicated to observe the behavior of the substructures electron-optically at the start of infection and to gain functional knowledge in this manner. Such studies can only lead to success when a large number of micrographs of high quality is available. Besides, possible biochemical or morphological changes would not decide whether individual cases involve a development within the scope of the infective process, or whether they are manifestations of lytic-degenerative processes to be expected in the enzymatic milieu of the host cell. Indications of such difficulties appeared quite early, when the study of pepsin hydrolysis revealed reactions deviating from normal, namely, empty membranes and completely unimpaired elementary bodies side by side with all transitional phases (87, 88, 63, 89). Among freshly prepared elementary bodies, e.g. those obtained from dab preparations, the number of abnormally reacting particles is usually higher than among purified suspensions. The divergent behavior of individual elementary bodies may be demonstrated with particular lucidity by means of preparations photographed prior to enzymatic attack. Fig. 13 a-b shows a particle at A, which is entirely normal prior to treatment with pepsin, but subsequently differs from others by the absence of an inner body. On the other hand, Fig. 10 contains two unimpaired particles near normally degraded elementary bodies. Mature elementary bodies consequently may differ radically in their biochemical composition despite identical morphology. The aforementioned results of proteolytic digestion of the dual element ("central density") also point in this direction. It is very probable that mature elementary bodies may manifest corresponding differences in functional performance.

The first insight is offered thereby into the genesis and decline of elementary bodies. Even more impressive is the demonstration of preliminary stages of the mature virus particle during ultracytological studies of infected cells. Following basic work by Gaylord and Malmick on the viruses of vaccinia, ectromelia and molluscum contagiosum (83) and the endeavors of particular technical perfection by Morgan and others with vaccinia and fowl pox viruses (84, 90), their results have been confirmed by several research groups (vaccinia 91-95, variola vera

60, molluscum contagiosum 96-98, fowl pox 99-101, ectromelia 102-103, rabbit fibroma 104-107). According to these findings, the processes leading to formation of mature elementary bodies are subject to the same laws among all viruses of this group. Based on investigations by our own research group of the propagation of vaccinia virus in HeLa cell cultures, undertaken in collaboration with G. Nielsen and K. H. Andres (94), the most essential results shall be described below.

The development of poxviruses apparently is restricted to the host cell cytoplasm. About 6-8 hours after infection, the first characteristic signs are seen --- frequently near the nucleus --- in the form of dense zones of variable extent, called "viroplasm" or "matrix." At the periphery, but often also in the interior of these zones, there are particles about 250 mμ in diameter, generally considered to be "immature" elementary bodies (Fig. 16 and 17). These bodies are delineated against the matrix or cytoplasm by a membrane of varying intactness and, surprisingly enough, show the same granular, fine structure in the interior as the socalled viroplasm. These bodies are most probably round; the oval structure reflected in the illustrations is a deformation ascribed to the cutting process. Preparations fixed with OsO_4 reveal that many of these particles possess a contrasting core surrounded by a light zone, usually in an excentric position (Fig. 16b). Owing to inadequate contrast, this structure is barely discernible in material fixed with $KMnO_4$ (Fig. 17, 20a). Nothing is known about the chemical composition and function of this body. The term "nucleoid," introduced by Morgan et al. (84) and accepted by most authors, has only hypothetical substance. There is some support for the view that the nucleoid is formed in the immature elementary bodies in the course of development. However, since every section through a virus particle does not necessarily bisect its nucleoid, this question cannot be decided immediately.

During the onward course of infection, in our system about 15 hours post infectionem, increasing numbers of mature elementary bodies are found initially intracytoplasmically and later, extracellularly, easily recognized by the characteristic internal structure (Fig. 16b). There is little doubt but that these develop from immature particles. The transition from one form to the other may take place with relative rapidity; structures that could be identified as positive transition forms are rarely seen. Deviations from normal are found also among the immature forms; their inclusion within the framework of this report was decided against, however, since no interpretation is possible at this time. The evaluation of such structures deals not only with developmental stages, but also with the results of lytic processes which affect every stage of development and, therefore, may lead to structural changes wholly unrelated to the normal course of viral synthesis. In addition, all possible structures may be masked by methodically imposed artifacts.

In the early stage of infection, the periphery of the formative centers consistently reveals large aggregates of mitochondria. Since these are intensely involved in metabolic activities, this fact is not surprising. This observation and the circumstance that immature elementary bodies are of approximately the same size as small mitochondria, led to the hypothesis that immature elementary bodies may develop from mitochondria (93). Our experience to date has failed to support this view.

Electron-optical studies covering the first 6 hours of the infective process have not yielded clues to virus-specific structures; a result that eminently fits the assumption of an ecliptic phase. Experimentally, this assertion is still marked by uncertainties, for it is enormously difficult to find one or a few virus-specific structures with the aid of sections only 1/500 cell diameters thick, in the limited field of a 20,000-fold magnification, especially since every cell cannot be assumed to be infected.

Now that the immature and mature elementary bodies have been recognized as two characteristic developmental stages of poxviruses, the question is raised whether the immature form is already infective. The fact that the infectivity in the system vaccinia virus-HeLa cell examined by us rises already 10 hours after infection, while mature elementary bodies are found only after 15 hours, seems to give an affirmative answer. It is possible, of course, that a few mature virus particles responsible for the increase in infectivity have escaped electron-optical detection. The question must therefore be held in abeyance. In addition, the significance of the so-called matrix requires critical elucidation. The assumption that this is an obligate structure preceding the elementary body, has not been confirmed satisfactorily, although it is quite likely, since such zones have been found among nearly all of the viruses of this group studied to date and in a wide variety of host cells.

Differences specific to the virus or host are discovered only in the later stages of infection, in agreement with earlier light-optical results; they appear with the formation of the classic inclusion body. A comparison of results involving molluscum contagiosum (Fig. 18) and fowl pox (Fig. 19), obtained in collaboration with K. H. Andres (see 108), reveals typical examples. In the former, the inclusion bodies seem to consist solely of mature elementary bodies. They are separated from each other by the trabeculae known from light-optics, which reveal numerous immature elementary bodies under higher magnification (109). It is not clear whether these trabeculae represent residues of the basic cytoplasmic structure or the so-called matrix. The inclusion bodies of fowl pox virus (Bollinger bodies), on the other hand, consist of a homogeneous, contrasting material in which mature elementary bodies are imbedded, often only on the periphery. The appearance of vacuoles in the interior of inclusion bodies, described by Herzberg and Kleinschmidt (101) in connection with the virus of canary pox, is also characteristic (Fig. 19).

The basic structure probably represents a virus-imposed lipoid reaction product of the host cell. The basic structure is rarely homogeneous near elementary bodies; it usually has a granular or vesicular makeup (Fig. 20b). The imbedded mature elementary bodies, fixed with $KMnO_4$ in the case of the material under discussion, show the typical brick shape in vertical and horizontal sections (Fig. 20c).

The present report is incomplete in many respects. However, it should indicate the kind of methodical equipment that is currently available and the extent to which morphology can contribute to the understanding of the nature of viruses. Regarding the application of such studies to other viruses, we stand on the threshold of a very promising development. Considering the fact that electron microscopy has already yielded extremely significant contributions to the viral etiology of tumors (cf. 45, 110), this approach may be expected to undergo a favorable evolution in the future. Insight into the basic phenomena of viral propagation, whose sequence has not been completely clarified in any instance, will be gained by electron microscopy only when ultracytological methods are developed that permit the observation of the important early reactions of viral synthesis.

Illustrations.

Fig. 1. HeLa cell after infection with adenovirus (Type 3), zones of density and elementary body aggregates in the nucleoplasma; fixed with $KMnO_4$; 13,000 X.

Fig. 2. Crystal of adenovirus (Type 3) in the nucleus of an HeLa cell, fixed with $KMnO_4$; 30,000 X.

Fig. 3. Elementary bodies of adenovirus (Type 3) after degeneration of the cell nucleus, partly still in crystalline arrangement, partly dispersed in the plasma of an HeLa cell; areas containing elementary bodies are surrounded by membranous systems; fixed with $KMnO_4$; 17,000 X.

Fig. 4. Elementary bodies of adenovirus (Type 3), internal structures and polygonal form are distinctly discernible; fixed with $KMnO_4$; 90,000 X. a) Intramuclear, in crystalline arrangement, b) intracytoplasmic, dispersed.

Fig. 5 a and b. Elementary bodies of influenza virus (A/Singapore) adsorbed by erythrocytes, fixed with formaldehyde, cores with lighter centers. Polygonal demarcation of elementary bodies evident, especially in a); fixed with $KMnO_4$; 130,000 X.

Fig. 6. Hemagglutination by incomplete elementary bodies (characterized by missing cores) of influenza virus (A/Singapore); the erythrocytic membrane, partly with dual contours, is clearly visible; fixed with $KMnO_4$; 130,000 X.

Fig. 7. Elementary bodies of vaccinia virus, prepared by the "critical point" method; the horizontally positioned particle on the left shows a central density and contrasting corner zones, the vertical elementary bodies on the right reveal dumbbell-shaped contrasting cores; fixed with OsO_4, (weak exposure); 65,000 X.

Fig. 8. Vaccinia elementary bodies after exsiccation from isopentane and oblique shadowing with uranium vapor; fixed with OsO_4; 65,000 X.

Fig. 9. Vaccinia elementary bodies with specific luminosity of inner body structure due to electronic irradiation following treatment with carbon tetrachloride; fixed with OsO_4; 35,000 X.

Fig. 10. Vaccinia elementary bodies after exhaustive degradation with pepsin (alcohol-acetic acid fixation, pepsin in 0.1 mole cysteine, pH 3.5). Unimpaired particles next to membranes with cores and some with cores and central densities. Arrow: Inner body of a vertically positioned particle in profile; uranium evaporation; 35,000 X.

Fig. 11. Proteolytic degradation of the detailed structures of vaccinia elementary bodies in relation to pH, following fixation with alcohol-acetic acid. White: Zone of digestion, black: resistant zone. Top: peripheral layer; center: discal to annular core; bottom: central density or dual element. (Pepsin digestion in the presence of 0.1 mole of cysteine.)

Fig. 12. Various steps of enzymatic degradation of mature vaccinia elementary bodies in horizontal (left) and vertical (right) position, partly vaporized with uranium; 60,000 X. a-d) intact elementary bodies; e-h) membranes with core and central density; i-l) membrane with core; m-p) exposed cores; q-t) membrane with central density or lateral dual element; u-x) empty membranes.

Fig. 13. Vaccinia elementary bodies fixed with OsO_4; 25,000 X. Identical fields before and after treatment with enzyme; the vertically positioned particle is labeled V. a-b) pepsin, pH 2.0, 0.1 mole of cysteine. The vertical elementary body reveals a core in profile. Particle V' initially stood on edge, but assumed a horizontal position during the reaction. Particle A was degraded down to the empty membrane. — c-d) papain, pH 7.0. Vertical particles showed two lateral bodies; their contrast is combined in horizontally positioned particles, producing the socalled central density.

Fig. 14. Vertical and horizontal sections through mature vaccinia elementary bodies after divergent fixation. a-b) OsO_4, c-d) alcohol-acetic acid, e-f) formaldehyde, g-h) palladium chloride, c-h) contrast enhanced with uranylic acetate; 150,000 X.

Fig. 15. Schematic representation of the mature vaccinia elementary body with horizontal, vertical and diagonal sections; about 120,000 X.

Fig. 16. Sections through HeLa cells infected with vaccinia; fixed with OsO_4; 45,000 X. — a) two matrical zones with imbedded immature elementary bodies, b) matrix with immature elementary bodies, partly with nucleoid; a few mature particles.

Fig. 17. Section through HeLa cell infected with vaccinia, formative center near the nucleus (N), surrounded by mitochondria, matrix, immature elementary bodies with barely visible nucleoids; fixed with $KMnO_4$; 40,000 X.

Fig. 18. Section through human skin containing a lesion of molluscum contagiosum, inclusion bodies consisting of mature elementary bodies separated by trabeculae. On the left, a nucleus; fixed with OsO_4; 6,000 X.

Fig. 19. Section through chorioallantoic membrane infected with fowl pox, contrasting inclusion bodies (Bollinger) with mature elementary bodies in the periphery, partly with vacuoles; fixed with $KMnO_4$; 6,000 X.

Fig. 20. Sections through chorioallantoic membranes infected with fowl pox; fixed with $KMnO_4$; 60,000 X. a) Matrix with immature elementary bodies, nucleoid barely discernible, b) periphery of an inclusion body, granular substrate, mature elementary bodies, c) same as b) arrows point to mature elementary bodies with nearly horizontal sectional surfaces.