ONTOGENESIS OF HUMAN SMALLPOX VIRUS

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ABSTRACT

The vegetative stage of development of the human smallpox virus is described as being comprised of four successive periods: "without membrane" period; "open-cavity" period; "single-cavity" period; and "double-cavity" period. This classification is based on the organization of the virus membranes. This paper presents a description of the changes in virus structure during each period. It shows the strict sequence of morphological continuity and succession of differentiation of viral components. The most important stage of the development, the differentiation of virus nucleoid, takes place in the virus cavity morphologically isolated from the cellular *milieu* and limited originally by the external membrane of the virus and then by the second one, the primary membrane of the nucleoid. Osmiophilic fibrils 20 to 25 A wide, which fill the cavity of a mature nucleoid, become clearly evident after staining with uranyl acetate and are revealed only at the end of the last or double-cavity period. The main features of the developmental cycle of human smallpox virus may probably be characteristic of certain other groups of viruses.

INTRODUCTION

It is known that morphological differentiation of viruses of the smallpox group (ectromelia, molluscum contagiosum, vaccinia, Shope rabbit fibroma, smallpox of man, birds, sheep, swine) occurs during the process of their maturation (1–8).

The most detailed description of the developmental cycle of vaccinia virus may be found in the well known paper by Dales and Siminovitch (8). The present paper deals with investigations on the ontogenesis of human smallpox virus.

MATERIALS AND METHODS

Strains of human smallpox virus were isolated (by the authors in collaboration with Altshtein and Kirillova) from patients with variola vera and grown in primary trypsinized *Macaca rhesus* kidney tissue cultures. The virological characteristics of the strains in question were earlier published (9, 10). A continuous culture of cynomolgus monkey heart cells was grown in 60 mm Petri dishes in Medium 199 supplemented with 10 per cent native bovine serum at pH 7.5. On the 3rd day of growth the cultures of cynomolgus monkey heart cells were inoculated with 10^5 TCD_{50} of virus per Petri dish. After inoculation the virus was propagated in serum-free Medium 199. At 6, 12, 24, 48, 72, and 96 hours after inoculation, samples of the cultures were prepared for examination in the electron microscope by the methods used in our laboratory (11).

Sections 100 to 300 A thick were obtained with an LKB ultratome, were stained with 3 to 5 per cent uranyl acetate for 2 to 3 hours or with 0.5 per cent $KMnO_4$ for 30 minutes, and were examined in a YEM 6c type electron microscope with instrumental magnifications of 18,000 and 56,000.

RESULTS

Preliminary selection and orientation of single cells made it possible to correlate the data from light microscopy with the results of electron microscopy and to study successive stages of the pathological process. The main results are summarized

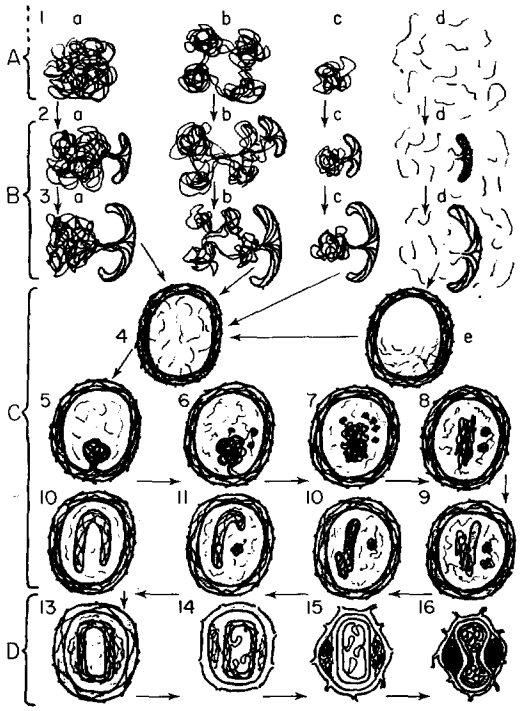


DIAGRAM I ontogenetic periods of human smallpox virus.

A. Without membrane period

1a, dense matrix; *1b*, reticular matrix; *1c*, micromatrix; *1d*, loose matrix.

B. Open-cavity period; *2* and *3*-formation of primary membrane of virus.

C. Single-cavity period, showing successive changes within the virus cavity (*4* to *12*).

5 and *6*, beginning of the formation of primary nucleoid from the inner leaves of the primary virus membrane.

7, primary nucleoid moves to the center of the virus.

8 and *9*, fibrils of the primary nucleoid acquire a linear orientation.

10 to *12*, successive formation of primary nucleoid membrane from primary nucleoid.

D. Double-cavity period.

13, formation of primary side bodies.

14, appearance of osmiophilic fibrils in nucleoid cavity.

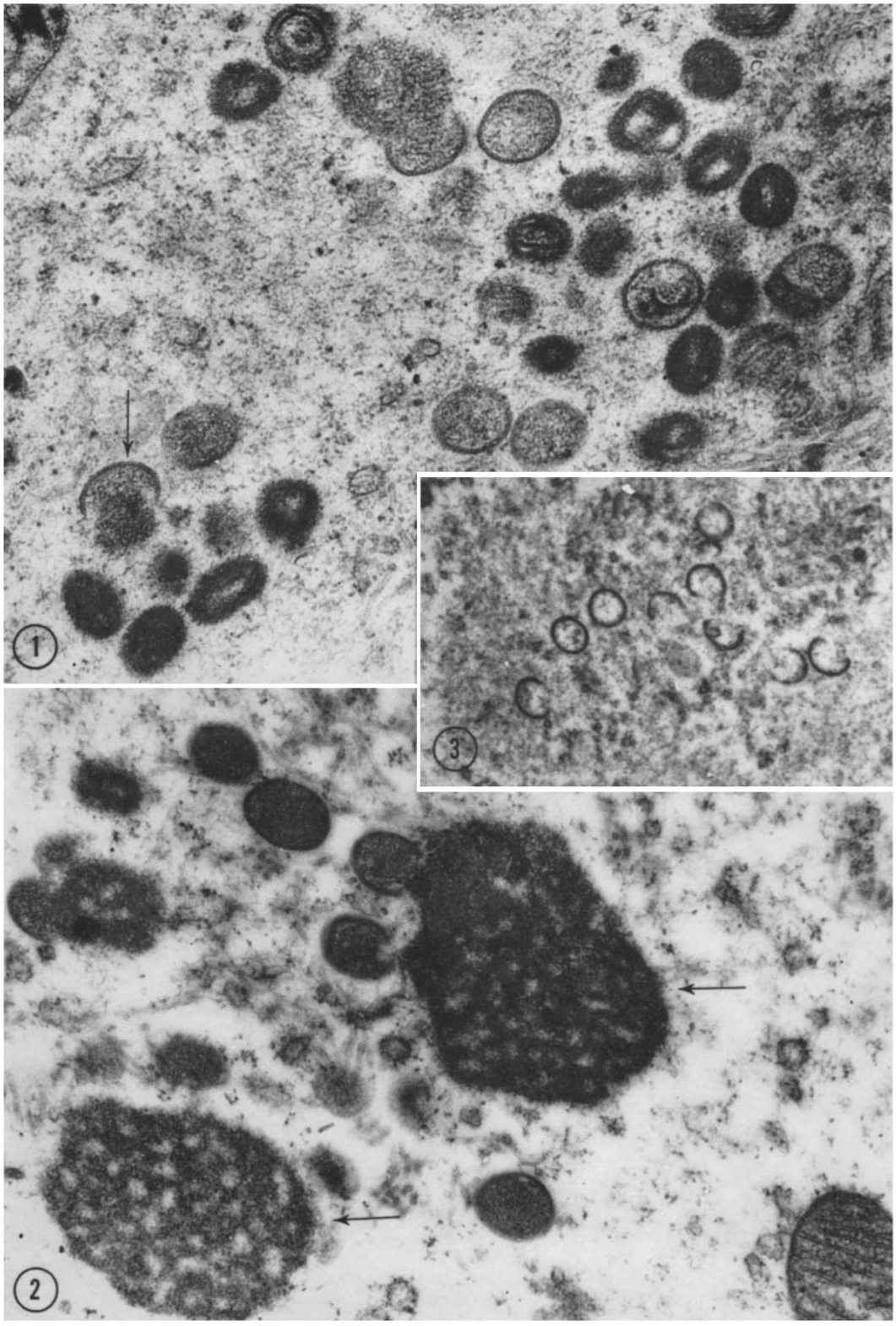
15, beginning of the changing of form of the virus and its nucleoid.

17, structure of mature virus. (The section passed through the maximum axis of the virus and the centers of two side bodies.

FIGURE 1 Part of tissue culture cell infected with human smallpox virus. On the right is seen a group of virus particles which are at different stages of ontogenesis; below and at the left is a micromatrix with a primary virus membrane fragment indicated by an arrow. Contrast enhanced with 5 per cent $KMnO_4$. × 50,000.

FIGURE 2 Part of tissue culture cell infected with human smallpox virus. The arrows indicate reticular matrix. Contrast enhanced with uranyl acetate. × 50,000.

FIGURE 3 Fragment of tissue culture cell infected with human smallpox virus. Portion of loose matrix. Contrast enhanced with uranyl acetate.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in Diagram I. In our scheme, the life cycle of each virus is divided into two stages: vegetative and spore (13). We have investigated the vegetative stage of smallpox virus development, which has four main periods: without membrane period, open-cavity period, single-cavity period and double-cavity period.

The first (without membrane) period of ontogenesis begins after the release of genetic information from the protein membranes of the virus which penetrates the cell during phagocytosis. This period ends with the formation in the cytoplasm of large zones of dense osmiophilic material called "matrix" (1) or "viroplasm" (4), and therefore it is the final stage of this period that can be studied by electron microscopy. The distinctive feature of this period is the absence of the viral membrane around the nucleoid (14).

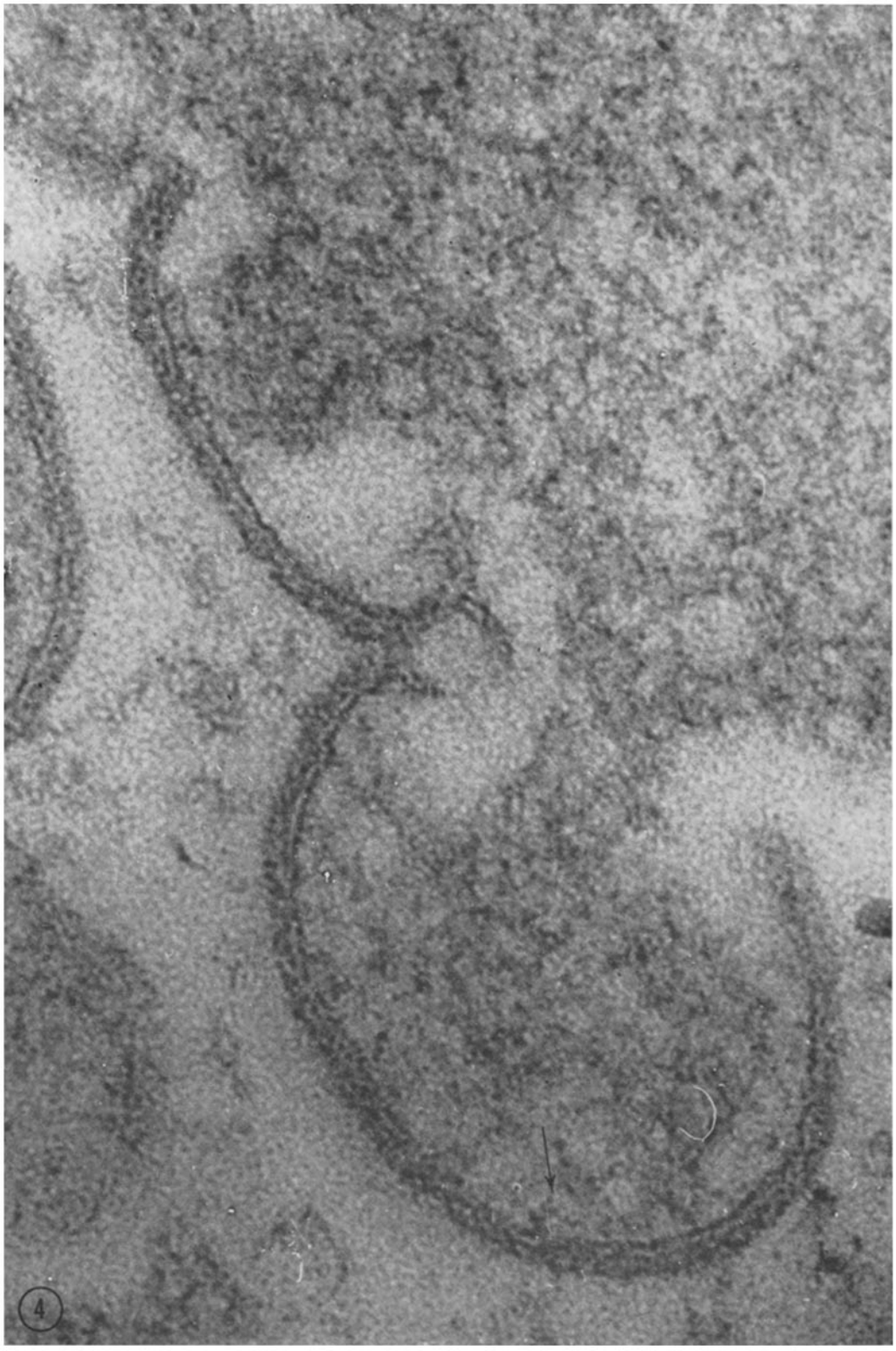
Four types of matrix were revealed: "dense" matrix (Diagram I, *1a*; Fig. 4), "reticular" matrix (Diagram I, *1b*; Fig. 2), "individual" or "micromatrix" (Diagram I, *1c*; Fig. 1) and "loose" matrix (Diagram I, *1d*; Fig. 1).

All types of matrix consist of randomly distributed osmiophilic fibrils 20 to 25 A in diameter which become more prominent after staining with uranyl acetate. In those rare cases where fibrils are favorably oriented for examination, each fibril is clearly seen to have a helical shape with a diameter of 40 to 50 A, and the distance between adjacent gyres is 40 A. It was possible to trace not more than 5 to 6 gyres per helix; thus, the total molecular weight of such a fragment probably does not exceed 4×10^5. The dense matrix may be localized in any part of the cytoplasm; its diameter does not exceed 1 μ and its density is uniform throughout. The reticular matrix consists of an aggregate of compact fibril sections connected with each other; the diameter of each section (a group of fibrils) is 300 to 500 A. The total size of the reticular matrix is the same as that of the dense one. The main feature of the matrix type described is the subsequent formation of virus protein membranes only on the surface of the matrix, not in its bulk. In this way the virus particles are formed in the dense and reticular matrix, and in each group the individual particles are at different stages of development (Fig. 1). The micromatrix has the same density as the two matrices described above. Its size, however, does not exceed 0.1 to 0.2 μ. One virus particle is formed from the micromatrix. All 4 types of matrix can be seen in one cell. They are often in proximity with one another. Mixed types of matrix were also observed (*e.g.*, dense and reticular in Fig. 2). The types of matrix described above may be considered as successive stages of matrix development. Finally, the fourth type, the loose matrix, is the largest in size and occupies areas about 3 to 5 μ in diameter (in the plane of one section). The loose matrix also consists of an irregular interlacement of osmiophilic fibrils 20 to 25 A thick; per unit of volume, however, the fibrils are fewer in number than in the other types of matrix described above. The main characteristic of the loose matrix is the subsequent formation of virus membranes (Diagram I, *2d*) throughout its bulk. Therefore, large aggregates of virus are formed from the loose matrix, the virus maturing more synchronously. The microcolonies consist of hundreds of virus particles all approximately at the same stage of development.

In the second (open-cavity) period of ontogenesis the "primary membrane" of the virus is formed. As has been mentioned, it appears in any part of the loose matrix, but strictly on the periphery of the other types of matrix. This membrane, which is 150 A thick, consists of 4 to 5 osmiophilic layers, each not exceeding 30 A in diameter. The layers are not always strictly parallel to each other, and they are often interrupted. It is possible to trace in certain sectors their direct connection with the osmiophilic fibrils of the matrix.

The size of the smallest among the revealed membrane fragments of human smallpox virus was 500 to 600 A. There is an inverse relationship between the size of any fragment (arc) of the forming membrane and the length of the circumference which corresponds to the arc; *i.e.*, the smaller the fragment, the bigger the circumference to which it corresponds. This means that the virus membrane while growing changes its shape as well. A peculiar bending of the membrane

FIGURE 4 Fragment of dense matrix with two primary virus membranes being formed. The monolayered structure of the membrane is seen. The arrow indicates one helical osmiophilic fibril. In this preparation (as in all the following ones) contrast is enhanced with uranyl acetate.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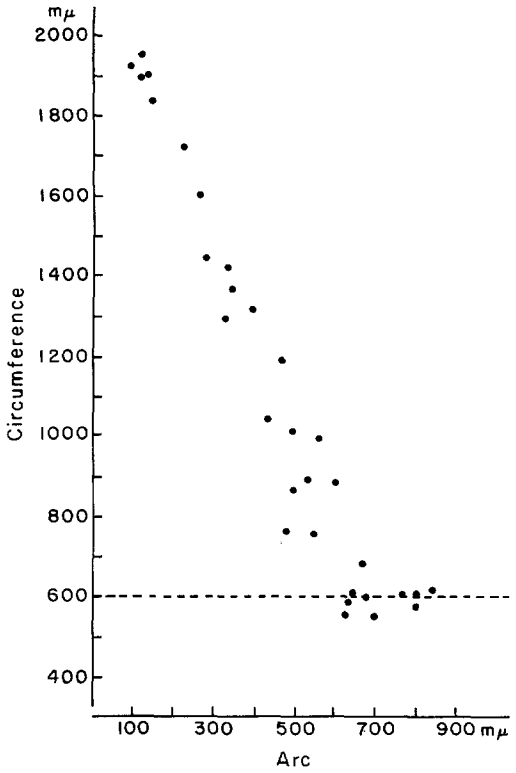


FIGURE 5 Graphic depiction of the phenomenon of uneven bending of the primary membrane of human smallpox virus.

occurs, the angle of bending decreasing gradually in the process of development. We call this phenomenon "a phenomenon of uneven bending of virus membrane." Fig. 5 presents graphically the phenomenon described. It was of interest that the curves which represent the phenomenon of uneven bending of virus membrane were identical for the viruses developing from all matrix types. Thus, the development of the virus membrane does not depend on the density of matrix. The primary membrane never penetrates the main bulk of the dense matrix; it is always located on its surface. The virus is connected with the matrix by means of a fibril bundle which gradually becomes thin, as though the substance of the matrix was absorbed mechanically into the virus cavity. The study of serial sections shows that the size of the "micromatrix" can be several times smaller than the volume of the virus which is formed from this micromatrix.

Virus membrane fragments were often seen to be localized at right angles and so close together that if virus particles formed by further development their contents would coincide almost completely. The final maturation of the virus in these cases is possible only under conditions of simultaneous (parallel) growth of both the primary virus membrane and the matrix from which it is formed. After the organization of the primary membrane is completed, the virus cavity is separated from the cellular environment.

In the 3rd stage, the single-cavity period, all the basic differentiation of virus structure takes place within the membrane-bounded virus cavity (Diagram I, *4* to *12*, Figs. 7 to 17). The virus body has a maximum axis of 3,000 A and a minimum axis of 2,500 A. The content of the virus cavity at first does not differ in structure from the corresponding matrix—a similar random interlacement of osmiophilic fibrils 20 to 25 A thick (Diagram I, *4*; Fig. 7) —and is called the primary viroplasm. Then at one point one or several layers of the virus limiting membrane invaginates and becomes spiral and protrudes into the virus cavity (Diagram I, *5*; Figs. 9 and 10). The fibrils within this invagination, which we call the "primary nucleoid," are more osmiophilic than the fibrils of the viroplasm, and their thickness is 40 to 50 A; *i.e.*, a 2-fold increase. Subsequently the primary nucleoid increases in size to a maximum of 1,000 to 2,000 A (Fig. 11), separates from the virus membrane, and becomes centrally located (Diagram I, *7*; Fig. 13). Sometimes an additional unusual structure is seen (Fig. 13, *A*). In the section this structure appears to consist of a chain of granules 90 to 100 A in diameter. The osmiophobic center of each granule is limited by an osmiophilic membrane 25 A thick. The granules may be pentagonal or hexagonal in shape. We failed to make a model of this structure.

As the size of the primary nucleoid increases, that of the viroplasm surrounding it decreases slightly (Figs. 14 and 15). The primary viroplasm retracts from the virus membrane but retains its close association with the nucleoid. The fibrils composing the primary nucleoid acquire more regular linear orientation and then gradually the primary membrane of the nucleoid is formed out of them, this new membrane developing in the same way as the virus membrane. The primary membrane of the nucleoid is about 150 A thick and consists of several osmiophilic layers, each about 30 A wide. During the single-cavity period the dimensions of the virus decrease to a maximum axis of 2,800 A and a minimum axis of 2,200 A.

The structure of the virus membrane remains unchanged.

After the formation of the primary membrane of the nucleoid is completed, another cavity appears inside the virus, and then the final stage, the double-cavity period, begins. The final differentiation of virus structure occurs during this period, the most important changes taking place inside the second virus cavity, under the nucleoid membrane. At first the nucleoid content does not differ essentially from the primary viroplasm: it consists of single fibrils, 20 to 25 A in diameter, which are oriented at random in the plane of the section. Then a more osmiophilic fibrillar component appears in peripheral sectors of the nucleoid, directly under its membrane (Figs. 18 and 19). In sections cut in a favorable plane these fibrils are linked with the inner leaf of the primary membrane of nucleoid.

Then the whole cavity of nucleoid becomes filled with numerous fragments of helical fibrils (Fig. 20). They are less osmiophilic than the fibrils of the primary nucleoid but are of the same thickness (40 to 50 A). The total size of the virus by this time decreases considerably; the maximum diameter is 2,600 A and the minimum is 1,800 A, the ratio increasing to approximately 1.5:1; *i.e.*, the same as that for the mature smallpox virus.

The maximum diameter of the nucleoid is 2,000 to 2,200 A and the minimum is 1,000 to 1,300 A. The configuration of the whole virus is changed too, the shape of the body resembling a straight parallelepiped with sharply rounded ends. The nucleoid also changes in configuration (Diagram I, *15*; Fig. 22). The side bodies play the most essential part in changing the virus shape, their bulk increasing considerably. As the result of the increasing pressure of the side bodies the nucleoid acquires the shape of a rectangular disc with rounded ends and two central invaginations.

FIGURE 6 Formation of primary virus membrane. × 207,000.

FIGURE 7 Beginning of the single-cavity period. × 207,000.

FIGURE 8 Part of primary virus membrane. The arrow indicates the place of transition of primary viroplasm fibril into the membrane of the virus. × 425,000.

FIGURE 9 Beginning of the formation of primary nucleoid from the leaves of the virus membrane. × 210,000.

FIGURE 10 Higher magnification of lower portion of Fig. 8. The arrow indicates the transition of osmiophilic leaves of the primary virus membrane into primary nucleoid fibrils. × 425,000.

FIGURES 11 and 12 Enlargement of primary nucleoid. × 207,000.

FIGURE 13 Part of virus body with primary nucleoid and granular structure (*A*). × 425,000.

FIGURES 14, 15, and 16 Primary nucleoid fibrils arranged in more regular rows relative to the maximum axis of nucleoid. × 200,000.

FIGURE 17 Early stage of primary nucleoid membrane formation. × 200,000.

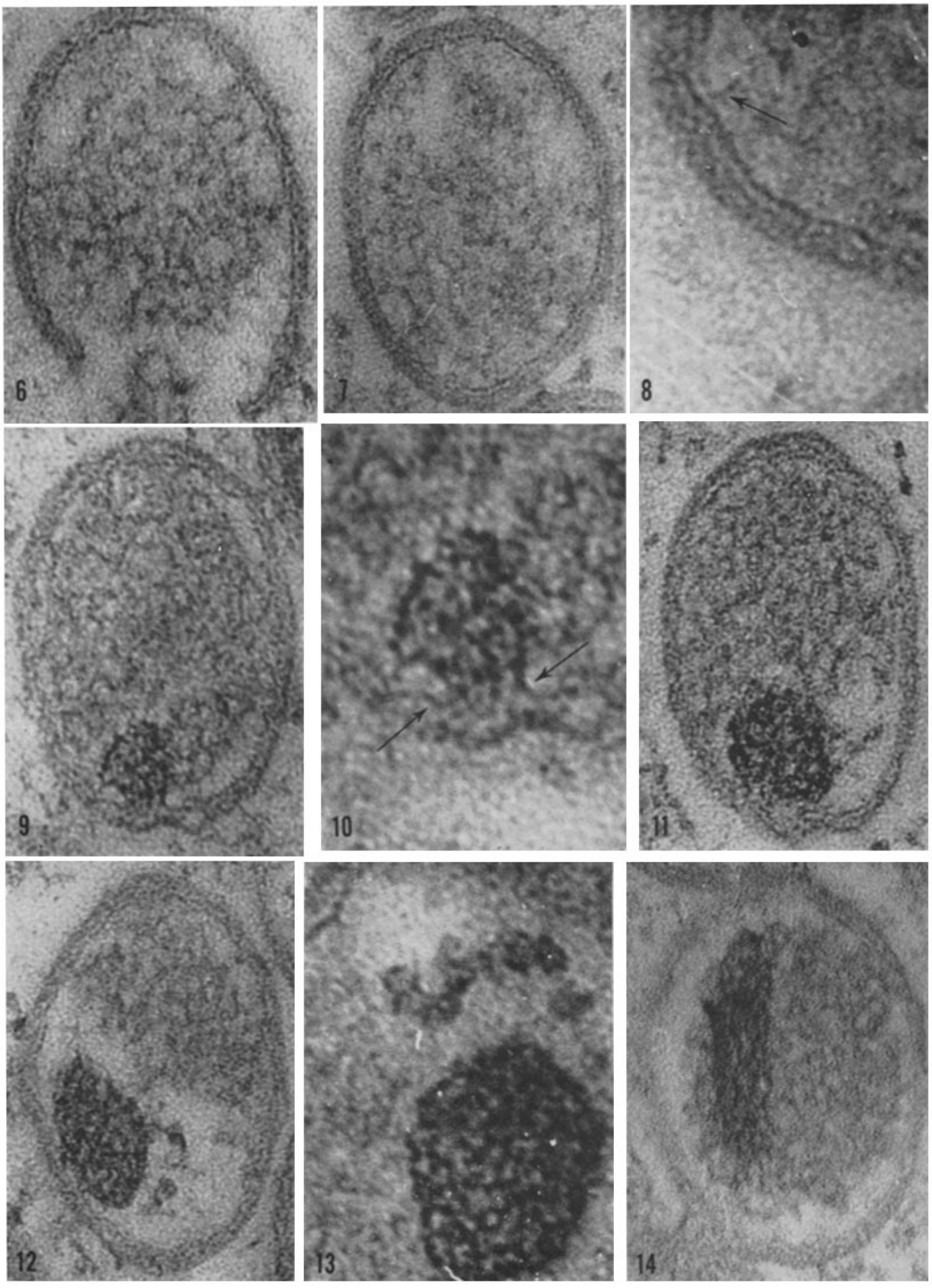
FIGURES 18 and 19 Initial forms of the double-cavity period.

FIGURE 20 The arrows indicate the formation of osmiophilic fibrils, 40 to 50 A in diameter, in the nucleoid cavity. × 410,000.

FIGURES 21 and 22 Enlargement of virus side bodies and changing of the shape of the virus nucleoid. × 220,000.

FIGURE 23 Structure of mature virus of human smallpox. The section passed through the maximum axis of the virus and the centers of the two side bodies. × 240,000.

For figures, see following pages.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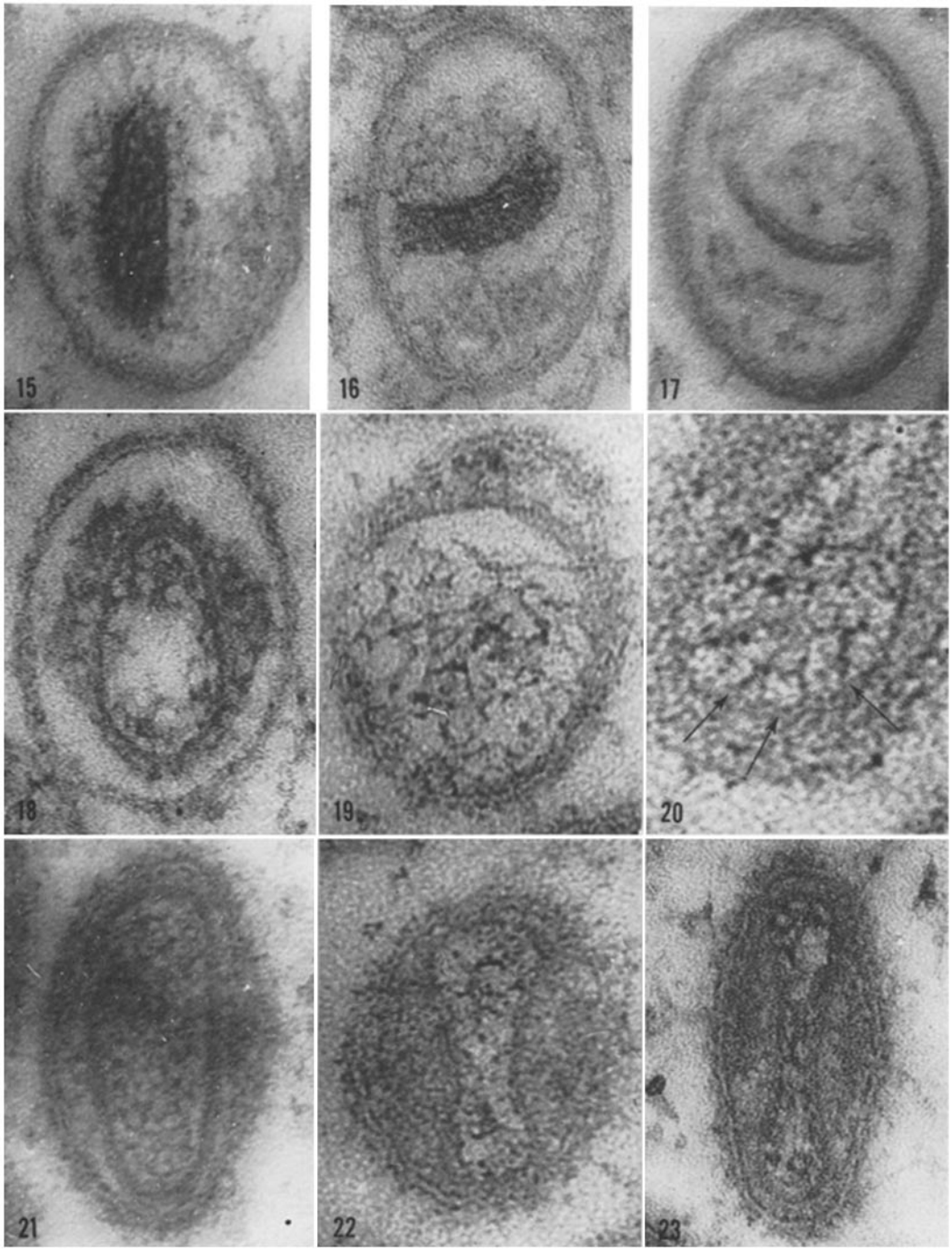
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Therefore the mature nucleoid comes to resemble a figure 8 in two planes and a rectangle with rounded ends in the third plane.

The external virus membrane also changes in structure, becoming three-layered (two osmiophilic layers 40 A each and a middle osmiophobic one 30 to 40 A) from a previous seven- and even nine-layered condition. The over-all diameter of this membrane is 110 to 120 A (Figs. 19 and 22). The osmiophilic layers are strictly parallel with one another. The mature nucleoid membrane (Fig. 23) also consists of three layers, its over-all diameter being slightly less (90 to 100 A). Fig. 23 shows the structure of a morphologically "mature" virus of human smallpox. The section cuts through the maximum axis of the virus and the centers of the two side bodies. The whole cavity of a mature nucleoid is filled with osmiophilic fibrils 20 to 25 A in diameter.

DISCUSSION

One of the most important questions to be discussed is the establishment of the degree of the validity of the developmental cycle of human smallpox virus described above. To prove this validity is difficult because the dynamics of the process can not be revealed by means of the electron microscope. Every investigator working in the field of electron microscopy is familiar with the difficulty of creating a model of any structure or a number of successive models of changing structure on the grounds of single static pictures. As a rule, when studying the developmental cycle of the virus, investigators try to achieve synchrony of cytopathic effect by means of very high infecting doses of the virus (100 or more particles per cell). We failed to obtain complete synchrony, however. The present paper will not consider in detail the causes of these failures.

But even the complete synchrony of the process of infection at the level of the whole population of cells under study does not mean the synchrony of the process of infection at the level of one cell. Moreover, the process of virus maturation and the dynamics of the cytopathic effect may not be temporally related. The virus colonies which localize in different parts of one cell are at different stages of development. Therefore, even when achieving synchrony of cytopathic effect of the virus, it is necessary to use other means for studying the dynamics of viral ontogenesis. As has been mentioned, we selected and oriented single cultured cells of different stages of infection by means of a phase-contrast microscope and then studied the successive stages of the pathological process by means of electron microscopy. In this way we have attempted to study virus differentiation and have obtained a continuous series of images showing the successive formation of virus components. From this study we have established that virus components themselves are differentiated asynchronously, enabling us to visualize the various stages in virus ontogenesis.

The main features of the developmental cycle of human smallpox virus coincide with the results of studies by authors who have investigated viruses of the smallpox vaccinia group (1–8). However, in our opinion, some new salient features are demonstrated in the present paper which are very important. The continuity and sequence in the maturation of virus components is shown: first the external virus membrane forms, and this is followed by formation of the nucleoid membrane, the side bodies, the inner structures of the nucleoid, etc. The time required for the maturation of each component varied. According to our scheme the external virus membrane takes three periods to form, the nucleoid takes two final periods, while the primary nucleoid takes but a part of one period (single-cavity period).

It should be noted that the data presented here suggest that the development of the nucleoid and side bodies takes place in a membrane-bounded virus cavity, and that the "mature" nucleoid, which consists of osmiophilic fibrils 20 to 25 A in diameter and is the place where the virus DNA is localized (8), is seen within the inner (second) virus cavity only at the end of the final (double-cavity) period of development. Tracing the morphological sequence of maturation of single components of virus has revealed that each step of the developmental cycle depends upon a previous one. The formation of a component of the virus is based upon the formation of a previous structure which in its turn is a foundation for the formation of a subsequent virus structure.

The primary membrane of the virus is formed from thinner 25 A osmiophilic fibrils, and the source for the primary membrane of the nucleoid is 40 A fibrils.

The primary membrane of the nucleoid becomes the membrane of the mature virus, while the inner leaf of the membrane is morphologically connected

with the fibrillar component which fills the nucleoid cavity. Consequently, all the viral components are formed from a morphologically homogeneous material, the matrix.

During the process of maturation the virus particle decreases in volume more than 2-fold (from 15×10^6 $m\mu^3$ in the single-cavity period to 7×10^6 $m\mu^3$ in the mature virus). The most significant decrease in volume occurs at the moment of formation of the primary nucleoid membrane.

Received for publication, November 11, 1963.

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