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## THE FINE STRUCTURE OF CYTOPLASMIC INCLUSIONS AND VIRUS PARTICLES OF BOVINE PAPULAR STOMATITIS

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The fine structures of cytoplasmic inclusions and virus particles of bovine papular stomatitis (B. P. S.) of one case were described. Two types of cytoplasmic inclusions, such as basophilic and eosinophilic inclusions, were observed in the epithelial cells. The former inclusions consisted of viroplasm and aggregates of a large number of virus particles which showed various developmental stages. The latter inclusions showed no evidence of virus particles and were subdivided into electron pale granular and highly opaque matrixes.

In the viroplasm, granular amorphous or core-like electron opaque structures (immature forms of virus particles) were enclosed by a shell which consisted of a unit membrane covered with a dense layer formed by subunits. The granular structures appeared to develop into a mature virus particle inside of a shell and finally the shell seemed to disappear.

### INTRODUCTION

Bovine papular stomatitis (B. P. S.) is caused by a paravaccinia subgroup of poxvirus<sup>1,15)</sup> and characterized by slightly elevated papules. It is often accompanied by erosions and ulcerations in and around the mouth without systemic signs of the disease<sup>13)</sup>. Cytoplasmic inclusions in the epithelial cells were reported by many investigators<sup>2,6,9,12)</sup>. RECZKO<sup>14)</sup> found virus particles in the epithelial cells of a methacrylate embedded section under the electron microscope.

Fortunately, the authors had an opportunity to conduct pathological examinations of one case of the disease in August, 1970. The animal was diagnosed B. P. S. on the basis of the existence of multiple papular lesions, cytoplasmic inclusions, and virus particles<sup>6,9,12~14)</sup>. The purpose of the present paper is to describe the fine structures of cytoplasmic inclusions and virus particles of B. P. S. in this case.

### MATERIALS AND METHODS

#### Calf

A male 32-day-old calf of Holstein breed was examined. The calf was

reared for beef at U-farm in Sapporo. He was killed by depletion of blood in our dissection room.

#### Histopathology

After careful macroscopical observations, the authors collected materials from various parts of the body. The materials were fixed with 10% formalin, and some pieces of the muzzle, the lips, and the buccal mucous membrane were fixed with Carnoy's solution. Paraffin and frozen sections were prepared. The sections were stained with hematoxylin-eosin, and the Carnoy's fixed sections were stained with Giemsa and methylgreen-pyronine. Feulgen and periodic acid Schiff reactions were also performed. Formalin fixed frozen sections were stained with Sudan III for neutral fat.

#### Electron microscopy

The materials were taken from the muzzle and buccal mucous membrane. Formalin fixed specimens, which corresponded to the lesions of cytoplasmic inclusions seen in the hematoxylin-eosin stained sections, were refixed in 1% OsO<sub>4</sub> for 1 hour. After dehydration through ethanol series, these specimens were embedded in Epon 812. Ultra-thin sections were double stained with uranyl acetate and lead citrate, and examined under HU-12A type and JEM-7 type electron microscopes.

### RESULTS

#### Clinical signs and gross lesions

Clinical signs started with diarrhea followed by disturbances of movement. The animal was unable to rise; at the time of killing he showed an extended, stiff neck (opisthonoid).

At the post mortem examination, multiple foci of exanthema were found in the muzzle, upper and lower lips and the buccal mucous membrane. Similar lesions were also found in the rumen, reticulum and omasum. These lesions consisted of rough, slightly raised nodules of rice grain to soybean size and were surrounded by a narrow zone of hyperemia. A few such lesions showed round erosions and formed a crater with a raised white border.

Macroscopical and histopathological findings of the other organs and tissues of this case will be described in a separate paper in the near future.

#### Histopathology

The epithelium of the nodules in the muzzle, the lips and the buccal mucous membrane showed hyperkeratosis. Erosions and ulcers with secondary bacterial infection were often found in the nodules. They were associated with hemorrhages and marked infiltration of neutrophils. Small and large areas of

hydropic degeneration and reticular colliquation were located in the spinous layer of the nodules. Nuclei in these areas often showed pyknosis and margination of the chromatin and karyorrhexis. The cytoplasm appeared empty. These degenerated cells contained one large or many small cytoplasmic inclusions of various shapes. The inclusions showed slightly basophilic or neutrophilic with hematoxylin-eosin stain and had homogeneous discrete and rounded structures (fig. 1). This type of inclusions was stained reddish purple with Giemsa, green with methylgreen-pyronine, positive for Feulgen reaction, negative with periodic acid Schiff reaction and negative with Sudan III stain. We tentatively called this inclusion "basophilic". The other type of cytoplasmic inclusions was also found particularly in the mass of spinous- and basal-cells adjacent to the areas of reticular colliquation (fig. 2). These cells were usually pale and swollen and often showed a slight degree of vacuolization. These inclusions appeared eosinophilic, granular, or in droplets and measured from 2 to 10  $\mu$  or more in diameter. We also tentatively called this inclusion "eosinophilic". Eosinophilic inclusions were also rarely observed in the degenerated cells with the basophilic inclusions. The eosinophilic inclusions were also found in the apparently normal epithelium, which was some distance from the nodules. The nodules showed hyperemia, inflammatory edema, and cell infiltration in the mucosae and the papillary bodies.

#### Electron microscopy

The cells of the spinous layer of the nodules had abundant cytoplasm. Bundles of tonofibrils were located at the marginal areas of the cytoplasm (fig. 3). Many virus particles and viroplasm were observed in the cytoplasm of cells with basophilic cytoplasmic inclusions seen in the hematoxylin-eosin stained sections (fig. 3). Viroplasm which consisted of homogeneous electron opaque materials included with immature virus particles appeared to correspond to the basophilic cytoplasmic inclusions (figs. 3 & 4). Furthermore, we could distinguish two types of matrixes in the cytoplasm. The one was electron opaque and was sharply demarcated (fig. 3), and the other was somewhat electron pale and granular and was widely distended (fig. 4). The matrixes themselves did not contain virus particles and may correspond to the eosinophilic inclusions in the sections stained with hematoxylin-eosin. Most of the electron opaque matrix was found particularly in the spinous and basal cells which had only eosinophilic inclusions, and these cells did not contain virus particles (fig. 5). But some of the electron opaque matrix was also found in cells with virus particles (fig. 3). On the other hand, the electron pale matrix was found only in cells with virus particles.

In the viroplasm, arch or circular membranous structures could be seen

(figs. 6~8). We tentatively named the membranous structures "shells", according to the terminology by TSURUHARA for similar structures in cells infected with Yaba poxvirus. The shell consisted of a unit membrane (7 m $\mu$  in width) covered with a dense layer formed by many subunits (ca. 11 m $\mu$  in length). Multiple arch structures of shell were also found in the viroplasm (fig. 6). Small spherical membranous structures were often located near the shell (fig. 7). These small membranous structures were ca. 60 m $\mu$  in diameter with a unit membrane ca. 7 m $\mu$  thick. Granular amorphous or core-like electron opaque structures were surrounded by circular structures of shell (ca. 300 m $\mu$  in diameter) (figs. 6~8). These granular structures were varied and seemed to represent various developmental stages of the virus particles. The shell also appeared to enclose a mature particle (fig. 8 b). The shell which enclosed another mature particle (fig. 8 c) seemed to disappear at the marginal area of the viroplasm, and the mature particle seemed to be released from the disappearing shell. The mature particles (figs. 7~9) were oval to cylindrical in shape (150 $\times$ 150 $\times$ 300~400 m $\mu$ ). The outer surface of the mature particles was covered with many projection-like structures of ca. 12 m $\mu$  interval (from center to center) in the ultra-thin sections (fig. 9). The viral core of mature particles looked like two circlets combined in the transversal section (fig. 9 a) and a pushed doughnut-like structure in the longitudinal section (fig. 9 b). As a three-dimensional structure, the viral core appeared to be a tubular structure in the shape of an ellipsoidal circular ring.

The nuclear contour was irregular and a margination of nuclear chromatin was conspicuous (figs. 1 & 10). In the nuclei of cells in which virus particles were found in the cytoplasm, various intranuclear structures could be seen. One of the intranuclear structures had aggregates of small particles which had a diameter of ca. 37 m $\mu$  and which were spherical in shape (figs. 10 & 11). Secondary structures were crescent-shaped electron opaque structures and sometimes appeared to be short tubules of ca. 100 m $\mu$  in diameter (figs. 10~12). They were aggregated in the nuclei. Tertiary structures were filaments which were observed near the crescent-shaped structures (fig. 12). These structures described above were never found in the nuclei of normal or uninfected cells.

#### DISCUSSION

In the present light microscopic observation, two types of cytoplasmic inclusions could be distinguished in the epithelial cells. Previous investigators<sup>2,6,12)</sup> have not given attention to the two types of inclusions which we have just described above. It appears that the basophilic inclusions may correspond to the B-type inclusions described by KATO et al.<sup>7,8)</sup>. They appeared to be at the site of viral maturation, since they were green for methylgreen-pyronine

stain and positive for Feulgen reaction and contained many virus particles of various developmental stages under the electron microscope. On the other hand, eosinophilic inclusions seen in hematoxylin-eosin stained sections appeared to consist of two types of matrixes under the electron microscope. The one was an electron opaque matrix and the other was an electron pale matrix. In both matrixes no virus particles could be seen. The exact nature of these matrixes is still unknown. But the electron pale matrix was observed only in the cells with viral multiplication; therefore, the matrix might have some relation to viral multiplication. This type of matrix may correspond to the A-type inclusions described by KATO et al.<sup>7,8)</sup>. Furthermore, the significance of the electron opaque matrix is controversial, whether it may be a precursory structure to the pale matrix or of a nonspecific nature found in viral infections.

Electron microscopic findings on the late stage of viral development of this B.P.S. virus were similar to those of Yaba poxvirus<sup>17)</sup> rather than vaccinia virus<sup>4)</sup>. The "shell", as we have described it, may correspond to the envelope surrounding the immature particle of vaccinia virus described by DALES & MOSBACH and the shell membrane of Yaba poxvirus described by TSURUHARA & TSURUHARA. From the findings of vaccinia virus described by DALES & MOSBACH, it seemed to us that they may regard the envelope as one of the structural components of the virus. TSURUHARA & TSURUHARA described that only subunits of shell membrane were removed at the latter stages of Yaba poxvirus maturation. In the present observation, however, the shell enclosed a mature particle and appeared to be an independent structure rather than a mature particle. The shell seemed to disappear at the marginal area of viroplasm. Therefore, the shell was not considered to be a structural component of the mature particle.

Many small spherical membranous structures — which may correspond to micelles<sup>16)</sup> — were also seen near the shell in our case. TSURUHARA described the micelles as structures corresponding to the unit membrane of the shell membrane and related to the formation of the shell membrane of the immature particles in the Yaba poxvirus. In previous reports<sup>14)</sup> on B.P.S. the existence of the micelles was not demonstrated.

The mature particles were covered with many projection-like structures. These structures are considered to be corresponding to the structures of peripheral tubular fibrils which were demonstrated by means of negative staining<sup>10)</sup>. The structure of these fibrils (crisscross pattern) was specific for the paravaccinia subgroup of viruses<sup>11)</sup>. The core of mature particle appeared to be a tubular structure in the shape of an ellipsoidal circular ring in the present observation. PETERS et al. described the viral core as triplet structures of strands in orf and

B. P. S. viruses. In order to clarify the truly fine structure of the viral core, a three-dimensional investigation may be necessary.

Reports on intranuclear structures in poxvirus groups are scarce. Electron opaque granules were reported in the basal cells infected with molluscum contagiosum<sup>5</sup>). Filamentous networks were also found in the epidermal cells infected with swinepox<sup>3</sup>). But crescent-shaped structures which we reported in the present paper were not found in any cells with pox of group viruses. It is very interesting to note whether these intranuclear structures are a secondary product of the virus infection or a viral structural component. DOURMASHKIN & BERNHARD thought that the intranuclear bodies of molluscum contagiosum have some relation with the virus infection. But recently it has become fact that the site of the synthesis of virus DNA and protein is not in the nucleus but in the cytoplasmic inclusion of B-type in the pox group of viruses<sup>7,8,16</sup>). CONROY & MEYER considered that the intranuclear filamentous networks in the swinepox infected cells were the results of an altered metabolic function and not a direct viral replication. Therefore, the significance of these structures may be a topic for further discussion.

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## EXPLANATION OF PLATES

### PLATE I

- Fig. 1 The spinous layer of the nodules in the muzzle  
The cells show hydropic degeneration and contain one large or some small basophilic cytoplasmic inclusions (↓).  
Hematoxylin-eosin stain × 650
- Fig. 2 Spinous- and basal-cells adjacent to the areas of reticular colliquation  
These cells are pale and swollen and contain granular (a) and droplet (b) eosinophilic cytoplasmic inclusions. Basophilic inclusions (c) are also seen in the degenerated spinous cells.  
Hematoxylin-eosin stain × 650
- Fig. 3 Electron micrograph of a spinous cell which contained basophilic cytoplasmic inclusion in hematoxylin-eosin stained section  
Bundles of tonofibrils are located at the marginal areas of the cytoplasm. A large number of virus particles, viroplasm (V), and electron opaque matrix (O) are seen in the cytoplasm. Aggregates of crescent-shaped electron opaque structures are seen in the nucleus. × 7,875

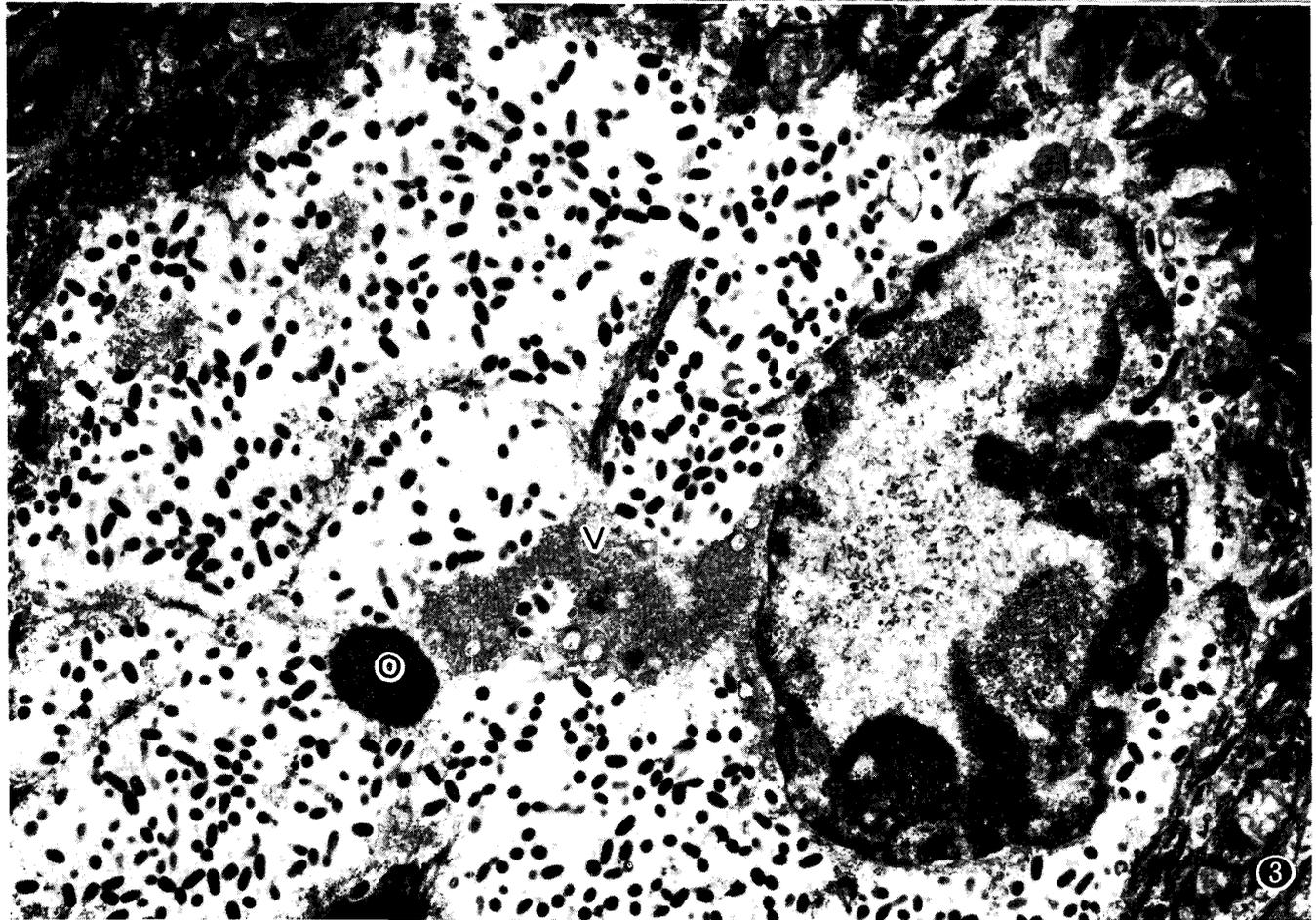
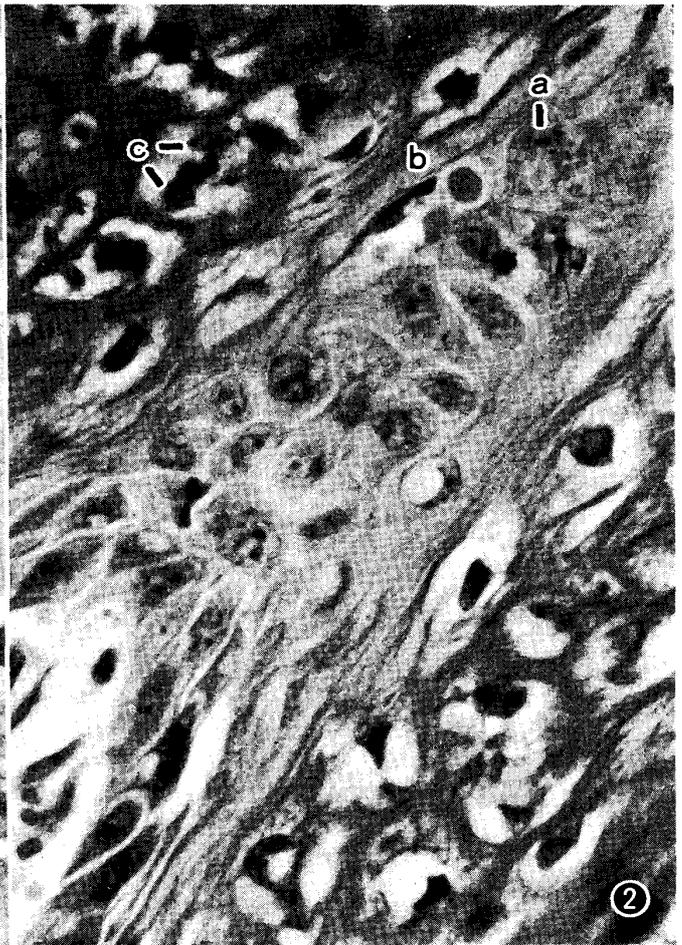
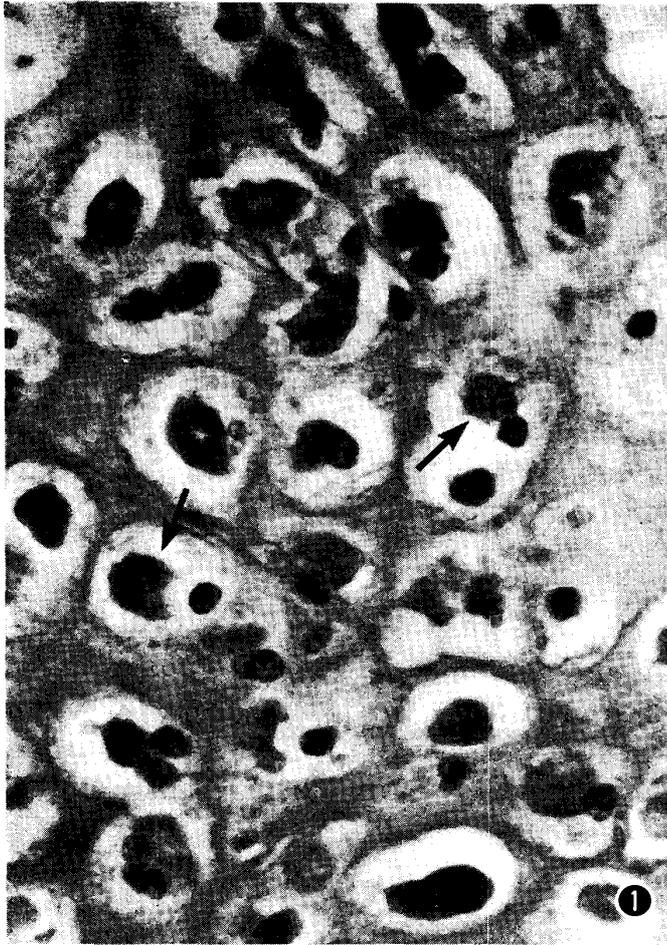


PLATE II

Fig. 4 Viroplasm (V) and electron pale granular matrix (P) in the cytoplasm

The viroplasm consists of homogeneous electron opaque materials and contains many mature and immature virus particles. Some marginal parts of the viroplasm (↓) appear highly electron opaque. Electron pale granular matrixes have no virus particles, but many virus particles in the other parts of the cytoplasm can be seen. × 7,875

Fig. 5 Spinous cells which had only eosinophilic inclusions in the hematoxylin-eosin stained section

Many electron opaque matrixes are seen in the cytoplasm. These cells have no virus particles. × 11,400

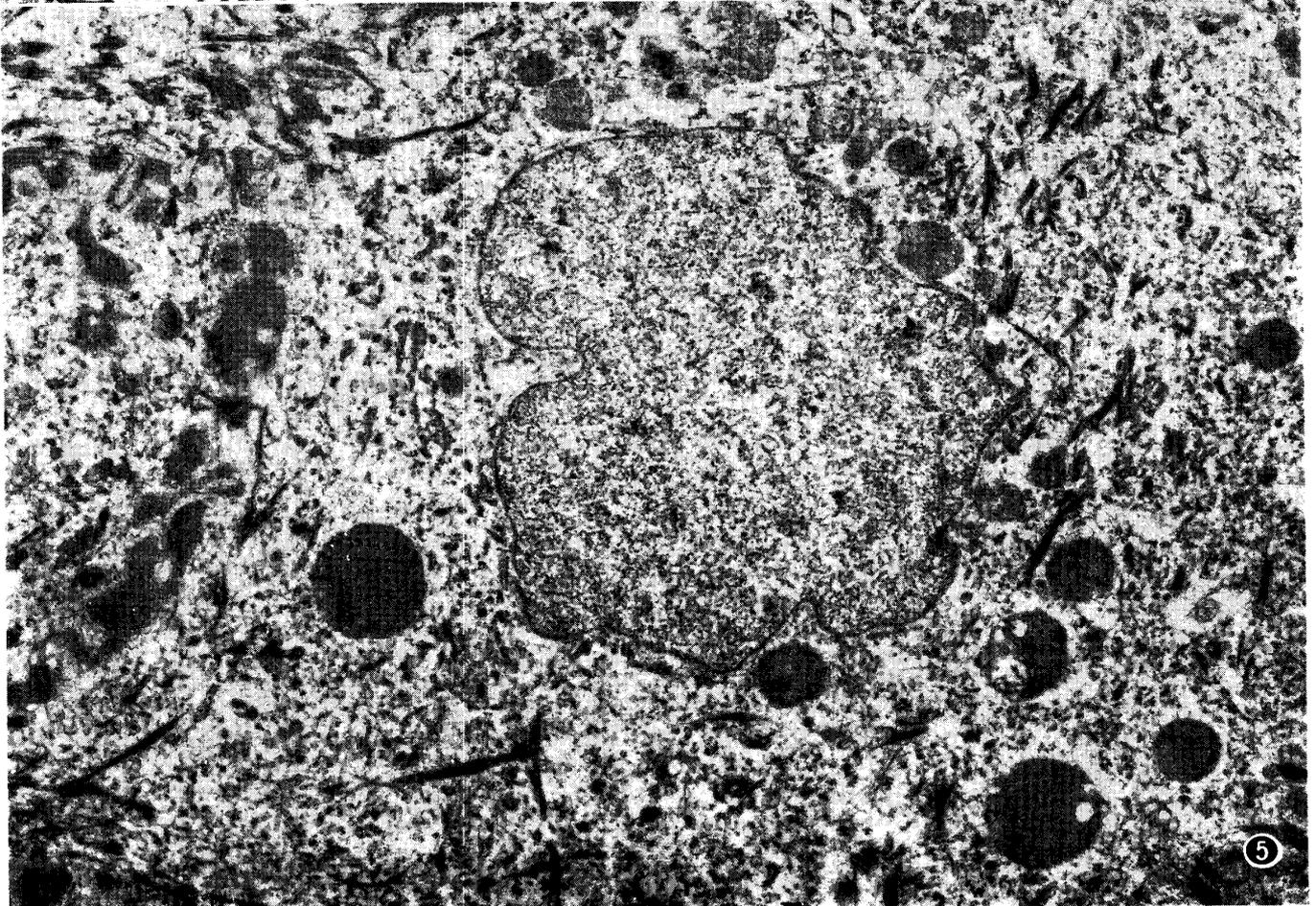
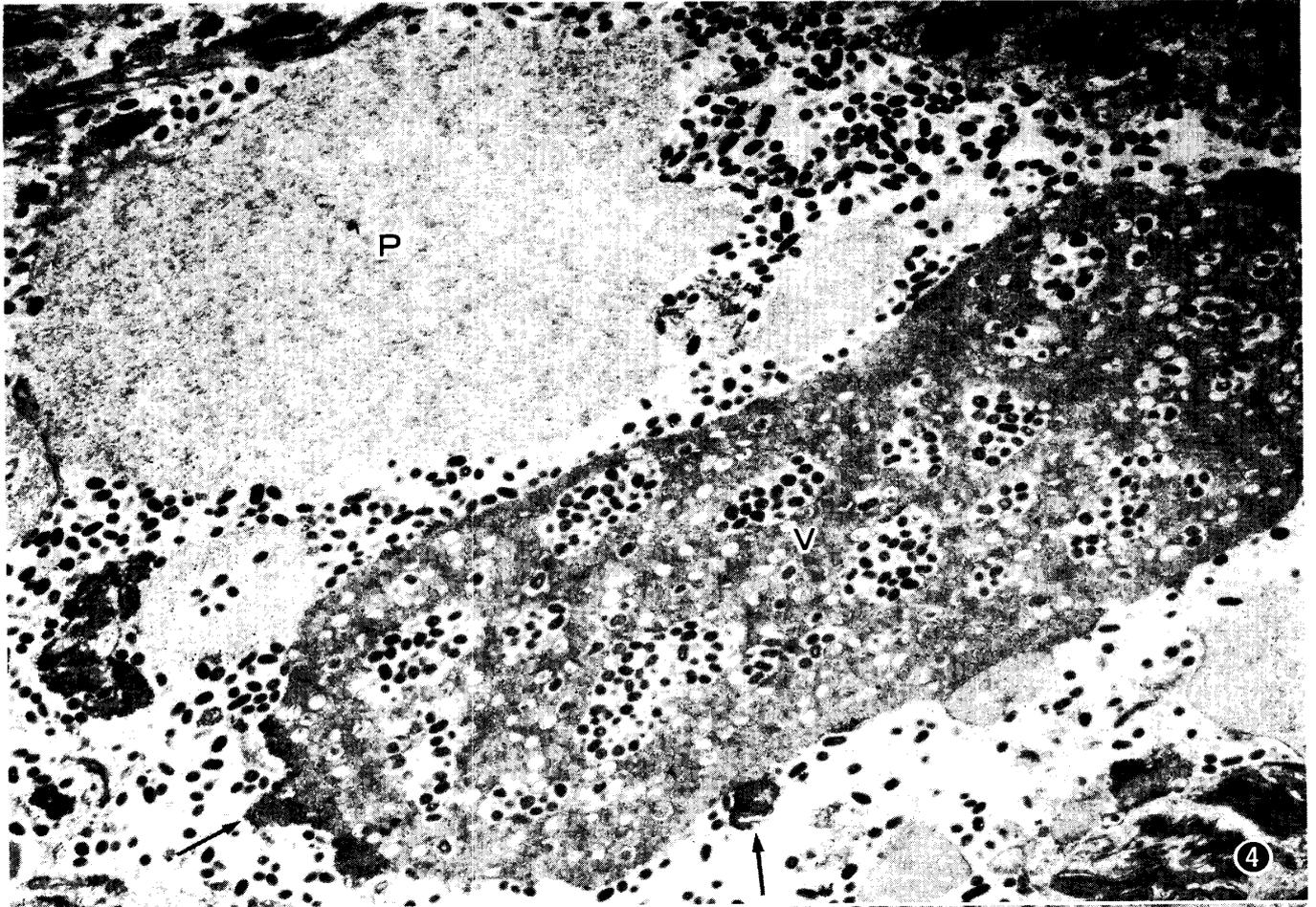


PLATE III

- Fig. 6 Immature form of the virus particles in the viroplasm  
Circular (upper) or multiple arch structure (lower) of shell enclosed granular electron opaque structures. The shell consisted of a unit membrane covered with a dense layer formed by many subunits ( $\downarrow$ ).  $\times 120,000$
- Fig. 7 Two small spherical membranous structures ( $\downarrow$ ) can be seen near the shell in the viroplasm. The shell enclosed core-like electron opaque structures. A part of a mature particle can be seen in the upper part of the figure.  $\times 120,000$
- Fig. 8 Virus particles of various developmental stages  
Centrally located shell enclosed granular electron opaque structures (a). The left shell enclosed a mature virus particle (b). A mature virus particle (c) in the bottom of the figure seems to be released from the disappearing shell at the marginal area of the viroplasm (V).  $\times 110,000$
- Fig. 9 Mature particles in the cytoplasm  
The outer surface of mature particle is covered with many projection-like structures ( $\downarrow$ ) of ca.  $12\text{ m}\mu$  interval on the ultra-thin sections. The viral core looked like two circllets combined in the transversal section (a), and a pushed doughnut-like structure in the longitudinal section (b).  $\times 110,000$

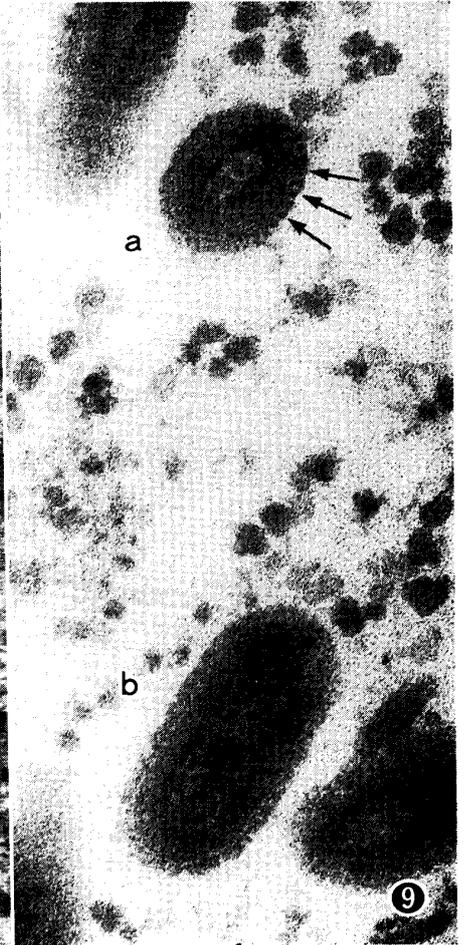
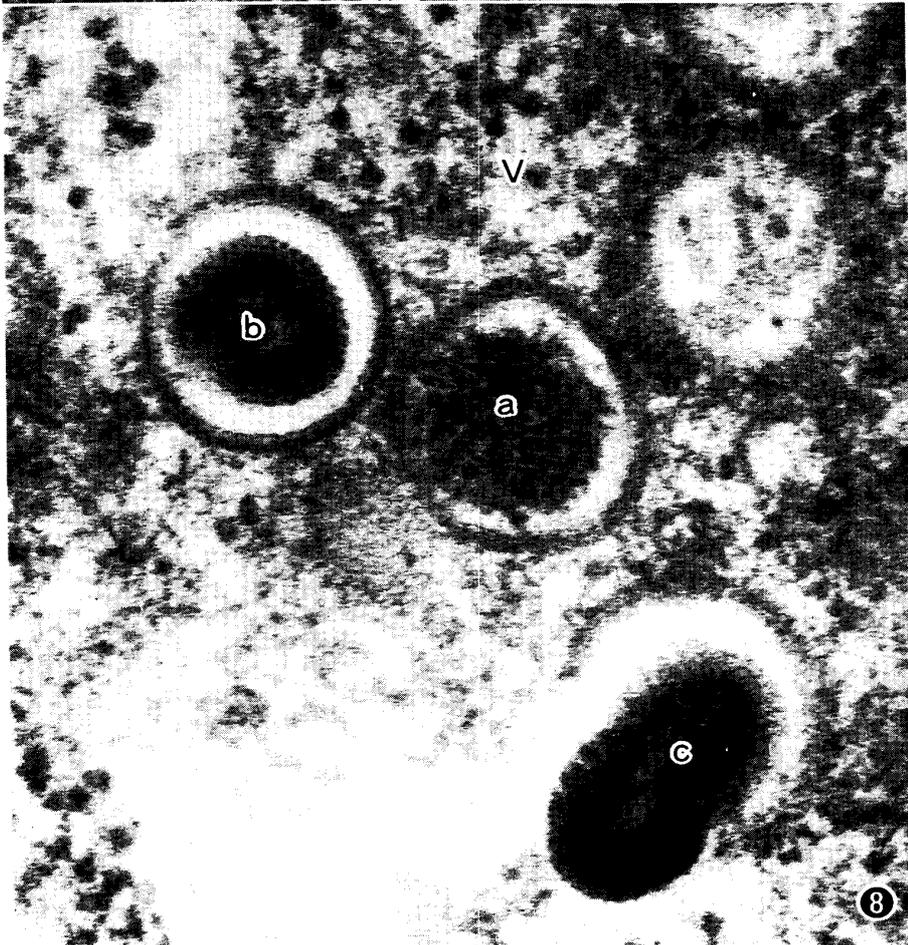
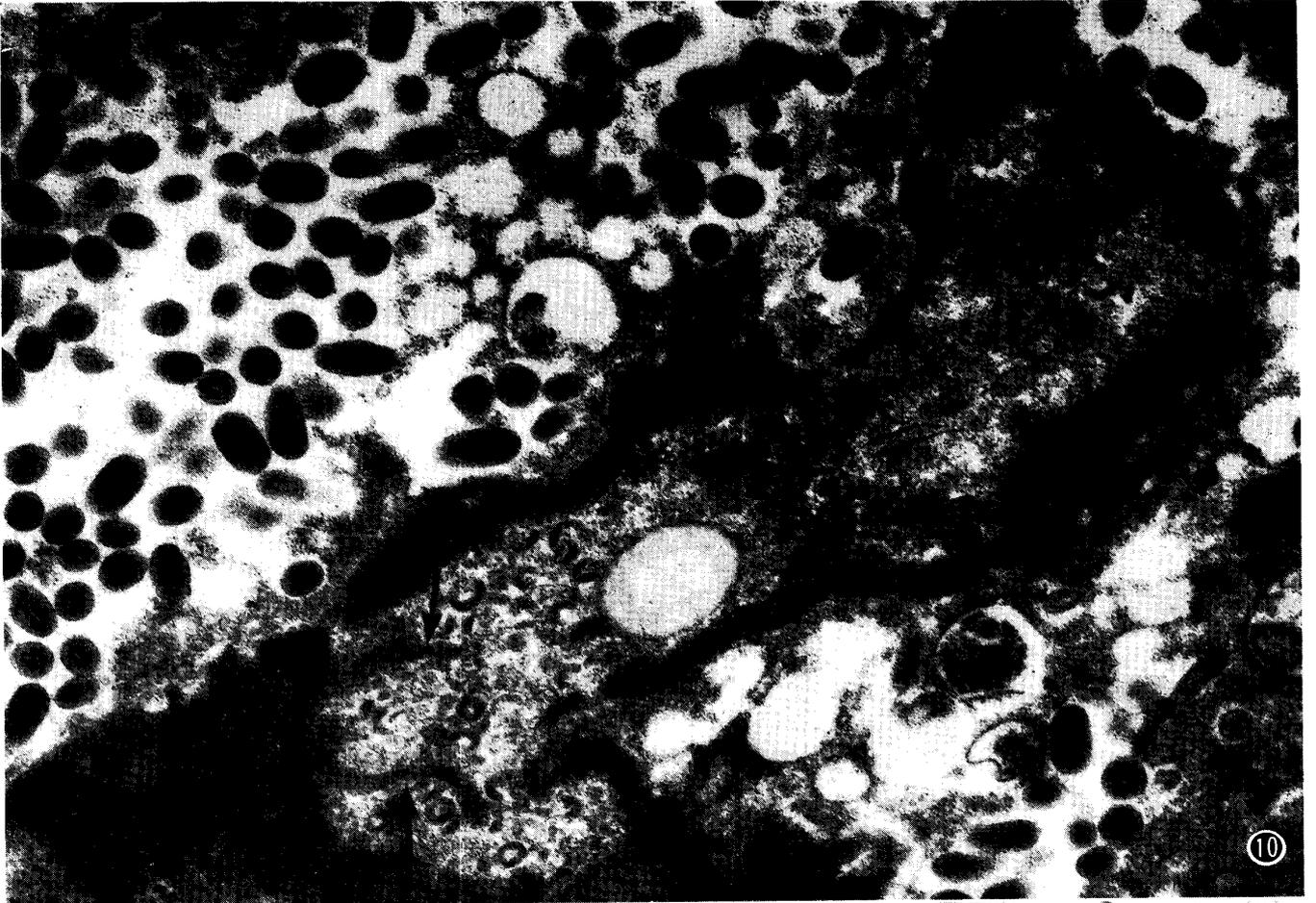


PLATE IV

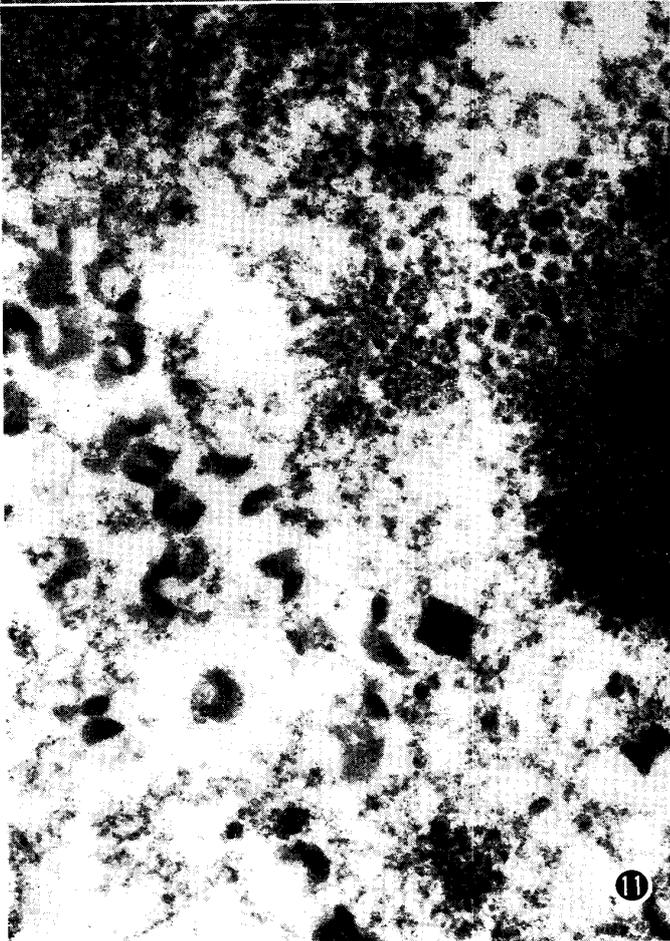
Fig. 10 Aggregates of small particles ( $\downarrow\downarrow$ ), crescent-shaped electron opaque structures and filaments ( $\downarrow$ ) are seen in the nucleus with a large number of virus particles in the cytoplasm.  $\times 31,500$

Fig. 11 Crescent-shaped electron opaque structures in the nucleus  
Some of them seemed to be short tubules. Small particles are also seen.  $\times 48,000$

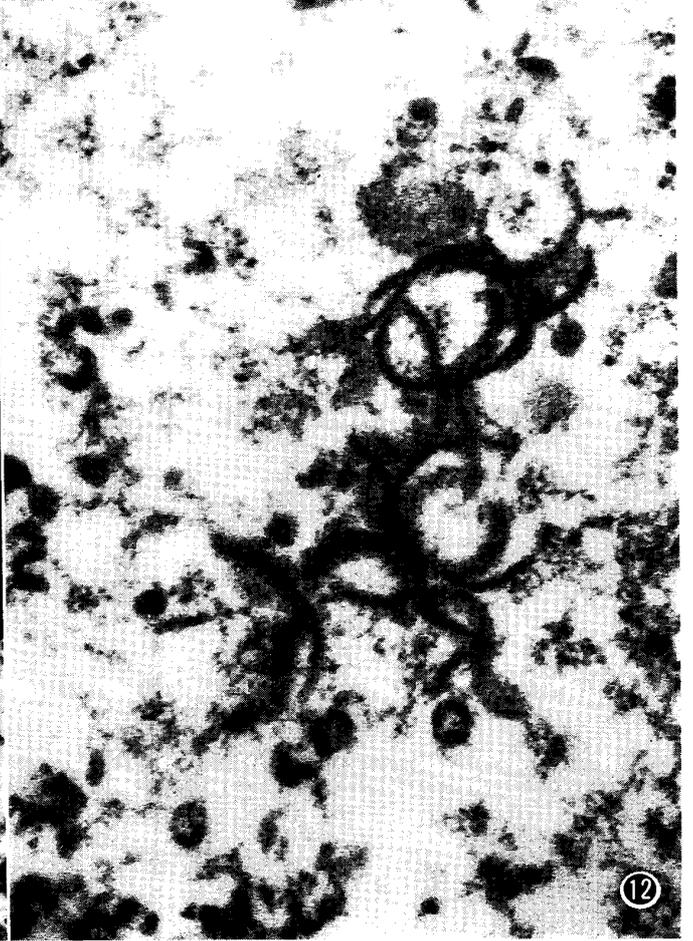
Fig. 12 Filaments are seen near the crescent-shaped structures in the nucleus.  $\times 48,000$



10



11



12