Production of the Milk Agent in Cultures of Mouse Mammary Carcinoma*

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PLATES 38 TO 41

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ABSTRACT

Thin sections of tissue cultures grown from tumors of the RIII high-breast-cancer strain mice were studied in the electron microscope. These tissues contain an abundance of particles whose morphology is consistent with biophysical measurement of the milk agent. These particles, found only extracellularly in our cultures, are formed at the cell membrane. The process of formation, as reconstructed from sections, appears to include a thickening and protrusion of the cell membrane which then evolves gradually into a dense sphere and separates from the cell in much the same manner as does influenza virus. The contents of the newly formed body are later rearranged to form a nucleoid within a membranous sac.

In 1948, Porter and Thompson (1) reported that an abundance of particulate bodies could be seen in unsectioned cells of epithelial sheets grown *in vitro* from explants of C3H tumors. Since these early observations, however, tissue cultures have not been used in the search for the specific particulate bodies representing the milk agent. From the literature (2–5) it is evident that particulate bodies have been found in regular association with the carcinoma cells; however, they were sometimes extremely scarce and in no way related to the degree of malignancy of the tumor. These bodies have been described as vesicles limited by an external double membrane with an internal dense core. The search for such structures in the cytoplasm of neoplastic cells has failed to give information on their origin, but new particles smaller in size (65 to 70 mμ) have come to the attention of several authors. Particularly well defined by Bernhard *et al.* (3) these particles, predominantly cytoplasmic, are characterized by a concentric double membrane. Because of their size, Bernhard assumed these structures to be the precursors of the "fully matured" extracellular particles and elaborated a theory for their formation (3 *a*).

The preceding article (6) correlates the physical, biological, and morphological properties of the milk agent and makes it possible to identify more confidently the active particles as seen in the electron microscope. Since many viruses proliferate better and are more easily observed in cell cultures, and because of Porter and Thompson's observation, we have used tumor cultures for a detailed examination of the milk agent and its formation.

Culture of Mouse Mammary Carcinoma.—Animals of the RIII strain of mice frequently show the three main types of mammary tumors with which we have been particularly concerned: (*a*) solid epithelial tumors with a lobular structure, (*b*) tumors with a hard, fibrous stroma, and necrotic areas, (*c*) hemorrhagic tumors. All three types can easily be grown *in vitro* with the conventional tissue culture methods.

Technique: After being aseptically removed, the tumors were washed in Simms' saline solution and cut up with a razor blade into approximately 2 mm. cube fragments. Fragments from a solid lobular

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tumor could be explanted as such; when the tumor was extensively fibrous and necrotic, however, only the tissue from the cortical zone was used. A good epithelial growth could also be obtained from hemorrhagic tumors if the red blood cells were first removed; washing in several changes of saline solution prior to explantation was therefore necessary.

The clean pieces of neoplastic tissue were then transferred onto a thick chicken plasma clot either in Maximow slides or in roller tubes and incubated at 37°C. There are no special nutritional requirements. We used a standard feeding solution composed of 3 parts of human placental serum (or horse serum), 3 parts of beef serum ultrafiltrate, 3 parts of Simms' saline, and 1 part of chicken embryo extract. This medium was renewed every 2 or 3 days.

Growth of the epithelium generally occurred on the 2nd or 3rd day following explantation. It appeared as thin sheets with a fairly regular border expanding concentrically around the tissue fragment as illustrated in Figs. 1 and 2. This epithelium, always very pure, grew rapidly, and sometimes in stratified layers. When fibroblasts developed as was often the case with fibrous tumors, each type of cell grew at a different level or in a definite portion of the culture area, without interpenetration. The maximum area covered by newly formed tissues was attained in about 10 to 12 days after which lysis set in. Groups of cells then became detached from the main colony and migrated to other parts of the culture vessel.

We have also studied (Figs. 6 and 7) cultures of a C57 mouse tumor which was induced by inoculating the mouse with extracts from embryonic C57 organ cultures (7). The organ culture was the last of a series of four culture transfers, the original of which had been inoculated with RIII milk. That is to say the agent from RIII milk was grown *in vitro* in tissue from a C57 mouse, then *in vivo* in C57 mouse, and then again *in vitro*, the last being studied here.

Electron microscopy: The cultures were removed from the Maximow slides or the roller tubes (by cutting out the plasma clot or scraping from the tube), placed immediately in Palade's (8) buffered solution of 1 per cent OsO_4 and fixed for 10 minutes at 4°C. The tissues were then washed in 50 per cent ethanol, rapidly dehydrated in 70, 95, and 100 per cent ethanol and embedded in deoxygenated butyl methacrylate (9). Blocks from the original tumors were also fixed and similarly embedded. Sections were cut with a Porter-Blum microtome (10), and examined in an RCA EMU 2 electron microscope.

Proliferation of Viral Particles.—At once a study of the thin sections lead to a fundamental observation: whereas few viral bodies were observed, and those only occasionally, in thin sections of fresh mammary tumors, a great abundance of them could be found in 5 to 14 day-old cultures of the neoplastic tissue. In marked contrast to observations on the tumor itself, tissue areas were seldom found that did not contain them.

The location and distribution of these bodies seem to be exclusively extracellular, being either at the cell membrane or in the intercellular spaces (Figs. 3 to 7); only the smaller vesicular bodies, already described by the authors mentioned above, are seen in the cytoplasm. The process of virus formation is believed to be illustrated in Figs. 3 to 5. The reconstructed sequence has the following steps, labelled alphabetically. First, there is a protrusion and thickening of the cell membrane. The incipient virus then assumes the shape of a hemispherical boss as at *A*, which later protrudes further and thickens into a complete dense sphere, *B*. The sphere then breaks away from the membrane as illustrated at *C*. Particles *D* have less dense centers, and particles *E* show a rearrangement of material which finally becomes, *F*, the nucleoid within the thin membrane, described in the preceding article (6). The micrographs of the preceding paper demonstrate similar images and the mechanism of virus formation appears to be similar to the way in which elementary bodies of influenza virus are formed (11). Benedetti and Bernhard (12) have observed a similar budding process possibly related to virus formation in erythroblastic leucosis.

It is of interest to mention that in earlier attempts to determine the location of the milk agent in cultures of mouse mammary carcinoma with Coons' fluorescent antibody technique, the writers noted that all cell boundaries were brightly outlined whereas the cytoplasm remained dark. The full significance of this preliminary observation was not then recognized because of uncertainty as to the specific nature of the antigen.

DISCUSSION

The great abundance of virus in tissue culture is probably due to the fact that the cells supporting virus production are grown selectively without the interference of fibroblasts, adipose tissue, and other

components of the glandular stroma. In a culture, following its normal course, all cells are active and very mobile. Necrosis is minimum. In actual tumors on the other hand, the cells are surrounded by a complex stroma, their protoplasmic movements are reduced and large areas of necrosis are present. The thinness of the sections and the high magnifications of the electron microscope, also reduce the chances of finding morphologically sound neoplastic cells. It is, therefore, not surprising, to find the viral particles only sporadically in tumor sections. Another reason for the high production of viral bodies in the culture may be the absence or reduction of defense mechanisms or the inhibitor postulated in the preceding article (6).

Intracytoplasmic Particles.—There is a question as to whether the 65 mμ vesicles seen in the cytoplasm (3) participate directly in virus formation. From our micrographs there does not seem to be any evidence that these vesicles are embodied within the virus at the cell membrane. As was pointed out by Morgan *et al.* (11), in the case of cells infected with influenza virus, these areas of dense vesicles may be related to cellular injury and may not represent viral inclusion bodies. The Golgi zone, which shows the greatest amount of these structures, is the region where the lamellar system is highly developed and where "inclusion bodies" often form. Similar areas have been found in influenza-infected mouse lung tissue (13), as well as in chorioallantoic membranes infected with vaccinia, fowl pox, and herpes simplex virus (11). The individual vesicles somewhat resemble isolated microsomes (14), but may not be identical with them. Their true significance must await further investigation.

Origin of the Viral Components.—The viral membrane seems to be identical with the cell membrane and is apparently formed from it as was believed by Bernhard (4), and as is demonstrated in many of our micrographs. The origin of the nucleoid, on the other hand, is not clearly demonstrated. By analogy with other viruses it is likely that the nucleoid contains nucleic acid which, in this case, would be an abnormal nucleic acid capable of transmitting malignancy. Although in many electron micrographs, RNP granules (15, 16) can be seen free in the cytoplasm, their inclusion in the virus is not apparent. It is possible that nucleic acid or nuclear protein of relatively low molecular weight accumulates on the cell membrane or is swept against it by cytoplasmic flow. Partially formed viral bodies are found either sessile or at the ends of microvilli, but in either case they are parts of spheres with radii varying little from 50 mμ.

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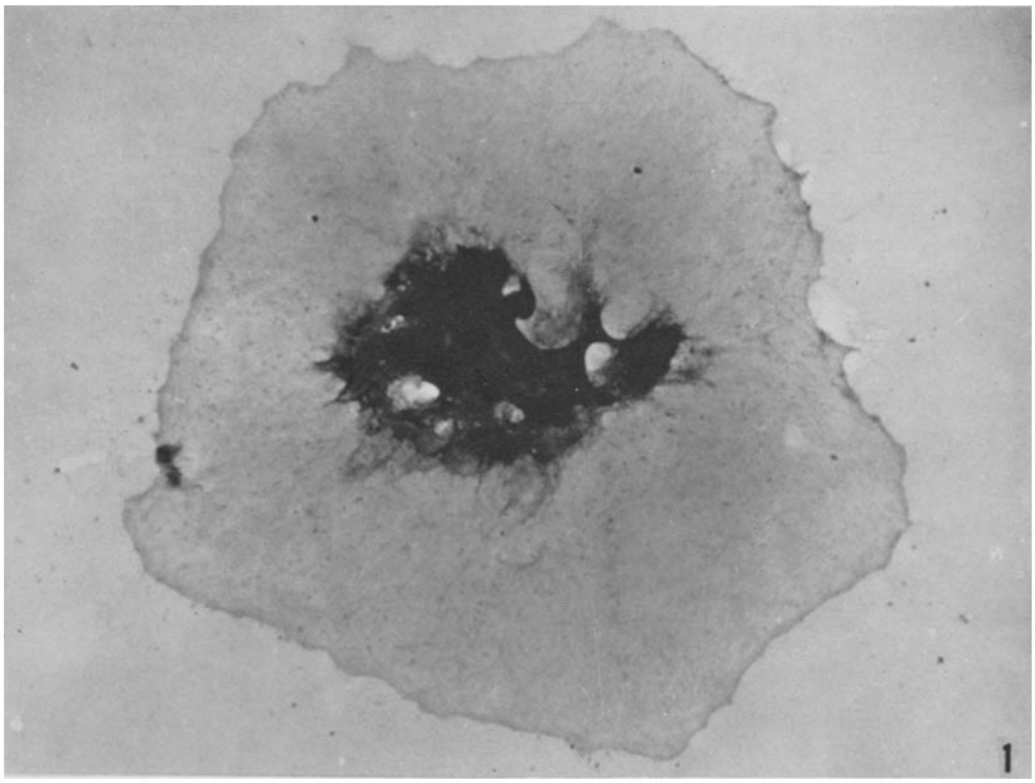
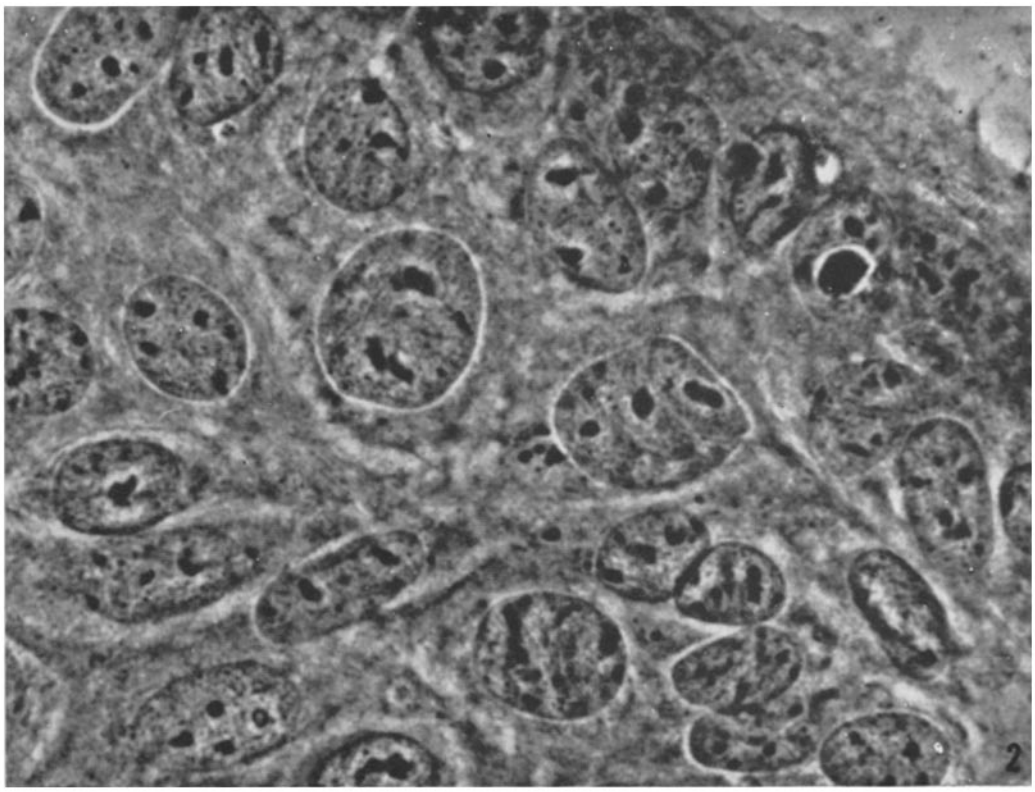
Explanation of Plates

Plate 38

Fig. 1. Whole culture of RIII mammary adenocarcinoma explant showing the regularity and purity of the epithelial outgrowth. This colony, 10 days old, has reached its maximal expansion. For electron microscopy it is detached from the culture vessel by cutting the plasma clot all around the outgrowth with a sharp blade, then lifting the whole culture from the glass with a small flexible spatula. After a short rinsing in phosphate buffer the tissues are processed for embedding in methacrylate. × 36.

Fig. 2. Margin of the same epithelial colony at a higher magnification observed in phase contrast microscopy. The nuclei are discrete with a markedly variable chromatin distribution and a sharply outlined nuclear membrane. In contrast the cell boundaries are ill defined and the protoplasm, granular in nature, does not show any specific structure. × 630.



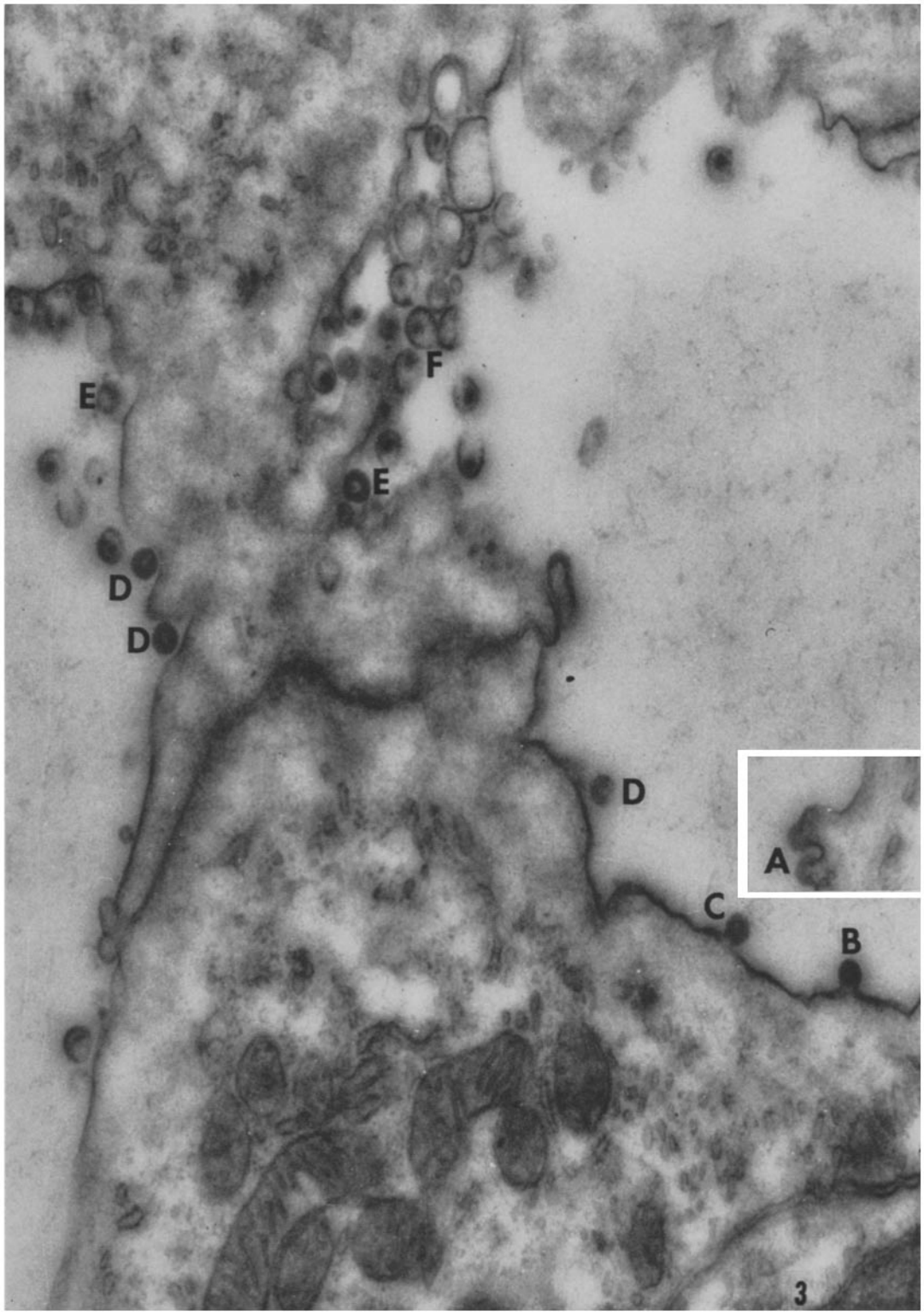


(Lasfargues *et al.*: Milk agent in mouse mammary carcinoma)

PLATE 39

FIG. 3. A 14-day culture of RIII tumor. A cell junction traverses an isthmus between two large intercellular spaces. Several types of particles are illustrated here. There are dense, solid bodies, hollow bodies with a dense shell, and those which consist of a dense core (nucleoid) within a thin membrane (sac). These appear to represent degrees of maturation. Their over-all dimensions are about the same although a few sacs appear larger. Most of the sacs show microtome distortion and some are ruptured.

The assumed process of formation is demonstrated along the membrane at lower right and in the insert taken from below the lower left corner of the micrograph. Two incipient particles appear at *A*. The dense cell membrane completely surrounds particle *B*, whereas *C* has just broken away leaving the cell membrane incomplete beneath. Particles *D* are all free in the intercellular space. The nucleoid may be in the process of forming in particles marked *E* and is completely formed in particles marked F. × 52,000.

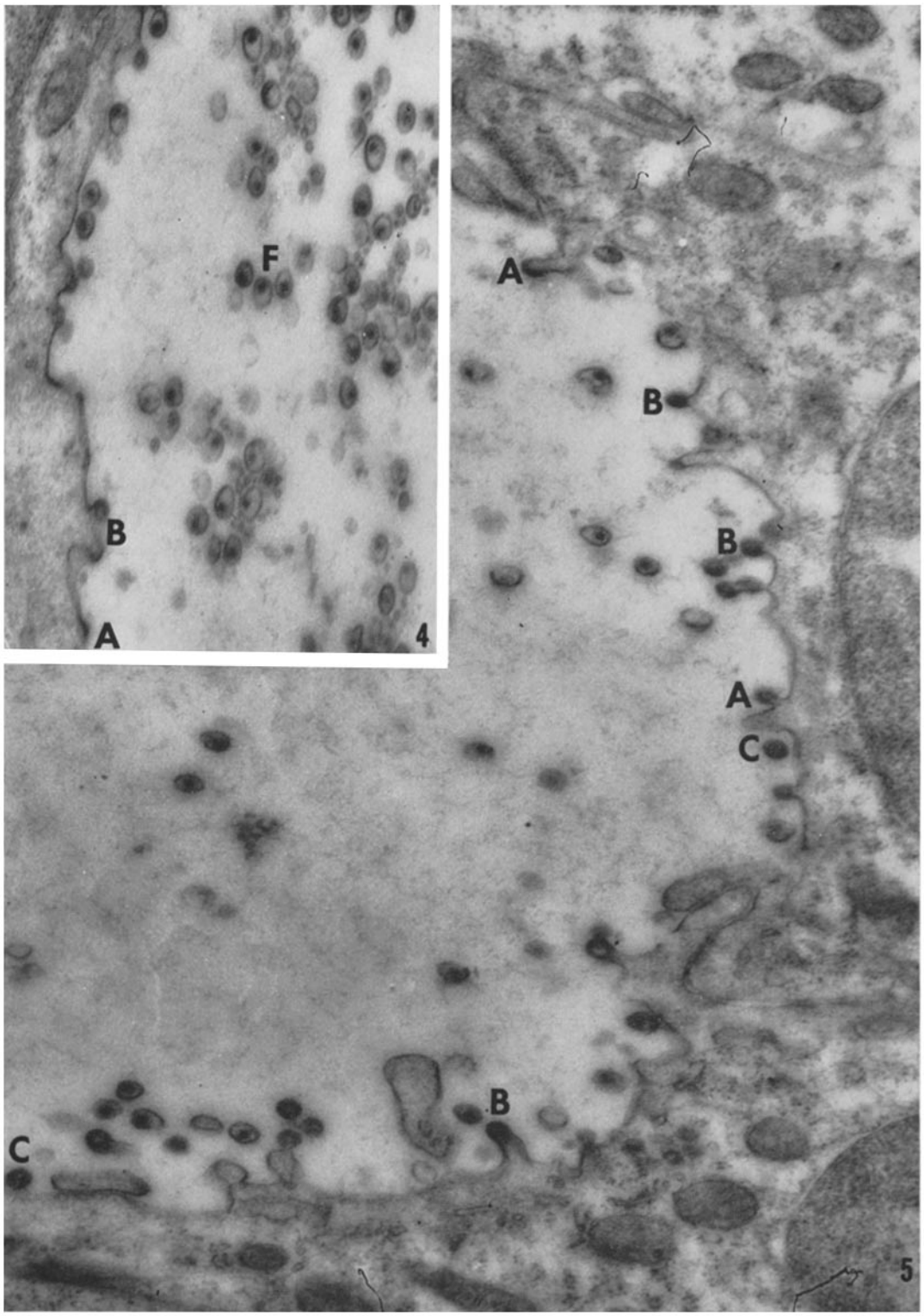


(Lasfargues *et al.*: Miilk agent in mouse mammary carcinoma)

PLATE 40

FIG. 4. Section from 14-day culture showing numerous virus particles. Stages of development are indicated by letters, *A*, *B*, *F*. × 41,000.

FIG. 5. Another section from same tissue as Fig. 4 illustrating the virus in many stages of its development, a few of them being labeled alphabetically, *A* to *C*. × 41,000.

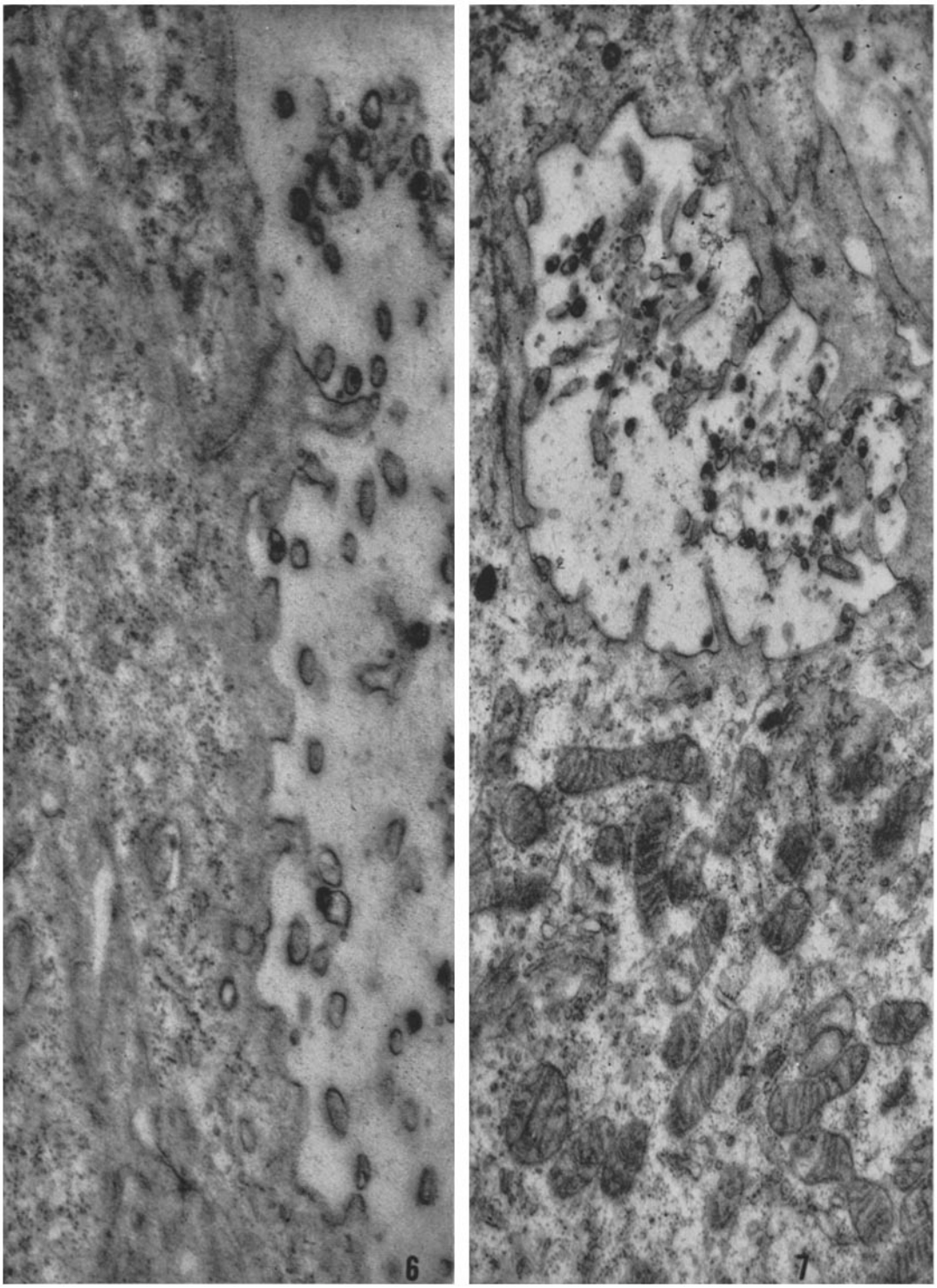


(Lasfargues *et al.*: Milk agent in mouse mammary carcinoma)

PLATE 41

FIG. 6. Section from a 5-day culture of tumor tissue taken from a C57 mouse which had been inoculated with the milk agent grown in organ culture of C57 embryonic breast. Numerous mature virus particles are evident. The cytoplasm at left contains many free RNP granules. × 41,000.

FIG. 7. Another section of the same tissue as Fig. 6. Mature viral bodies lie in the intercellular space and one or two can be seen forming at the cell membrane. Both free and bound granules are abundant in the cytoplasm. × 24,000.



(Lasfargues *et al.*: Milk agent in mouse mammary carcinoma)