FINE STRUCTURE OF THE HUMAN WART

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In the past 10 years, human warts have been the subject of several studies employing the techniques of ultrathin sectioning and electron microscopy. The state of preservation of the cells, however, as evidenced by the illustrative material, has generally been so unsatisfactory that it seemed worth while to re-investigate similar material by using recent technical improvements. Most notable among these improvements has been the Epon embedding procedure described by Luft.[1] In addition, techniques for staining sections, developed by Watson,[2] gave promise of better definition of the fine structure.

Beyond the need for better techniques, it was clear that several controversial points required resolution. Most prominent of these, perhaps, was the question of whether or not the virus-like particles associated with some warts occurred both intranuclearly and intracytoplasmically. Gaylord,[3] using a previously unpublished micrograph of Bunting, reported virus-like particles in the cytoplasm adjacent to the nuclear membrane. Although Gaylord regarded the nuclear membrane as intact, there were several sites of discontinuity which could account for the appearance of the particles in the cytoplasm. Charles[4] described a slight leakage of virus from the nucleus into the cytoplasm at a site of nuclear membrane rupture. The area discussed was unfortunately located just outside the figure reproduced. Williams, Howatson and Almeida[5] reported that no particles were seen in the cytoplasm of any of the cells they observed, a statement made earlier in the report by Bunting[6] in the first observation of virus-like particles in cells examined by electron microscopy. The present study will show that virus-like particles do occur, although rarely, in the cytoplasm of cells of the human wart, both in the granular layer and in parakeratotic areas.

Another disputed point concerned the degree of development of the desmosomes in cells of the malpighian layer of the wart. Gaylord[3] reported that there was failure to form intercellular bridges (desmosomes) as affected cells moved higher in the malpighian layer, an observation

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made earlier by Strauss, Bunting and Melnick.[7] Gaylord cited this as evidence indicating inhibition of normal cellular differentiation. Charles,[4] on the other hand, reported that prickles, or desmosomes, were developed to an abnormal extent in malpighian cells of the wart. He did not localize these as to their position in the malpighian layer. Charles's report is substantiated in the present study by the observation of several areas containing very dense accumulations of desmosomes.

Still another aspect of the situation requiring clarification is the question of the true size of the viruses or virus-like particles. Gaylord,[3] for example, reported a diameter of 68 mμ for separated particles in suspension, 52 mμ for packed particles in suspension, and 28 to 38 mμ for embedded and sectioned particles. Similar size ranges were reported by Williams and co-workers.[5] Siegel[8] reported finding a 16 mμ particle which he suggested was the causative agent. Oda[9] reported spherical or elliptical virus-like particles measuring 150 to 200 mμ in the stratum corneum and particles measuring 150 mμ in the prickle cell layer in human warts. In the light of these conflicting and varying reports, it seemed appropriate to determine the size of the particles following successful Epon embedding. A maximum diameter of 38 mμ was found.

There is also some question whether the nonviral intranuclear inclusions are truly intranuclear or whether they represent projections of the cytoplasm into the nucleus. Gaylord[3(p.467)] seemed inclined to consider applicable also to papilloma inclusion bodies the report of Kleinfeld, Greider and Frajola[10] that "thioacetamide-induced intranuclear inclusion bodies were actually cytoplasmic intrusions into the nucleus." The present observations suggest that these inclusions actually occur within the nucleus.

In conducting these studies, it was observed that a secondary infection of one of the warts by bacteria had occurred. As electron microscope observations of bacterial infection of cells or tissues are quite rare, several electron micrographs illustrating this phenomenon are included. These are particularly interesting because they demonstrate stages in bacterial cellular division.

Material and Methods

In studies of human warts, Lyell and Miles[11] distinguished two kinds: Type I (myrmecia), the deep or iceberg wart, in which both nucleus and cytoplasm contained eosinophilic inclusion bodies which first appeared in the cells of the prickle layer and increased progressively in size toward the surface of the skin. Type B (*Verruca vulgaris*), the superficial wart, in which there were no inclusion bodies.

The two warts used in this study belong to the first type. One wart was obtained, with 2 per cent procaine as a local anesthetic, from the right heel of a 16-year-old white girl. The second was obtained, without anesthetic, from the finger of a 22-year-old white man. Each specimen was fixed for 4 hours in 1 per cent osmium tetroxide,

buffered at pH 7.6 with acetate-veronal. The fixative was supplemented with NaCl, according to the recommendations of Michaelis.[12] After fixation the specimens were washed, dehydrated by passage through a graded ethanol series and embedded in Epon.[1] Ultrathin and thin (1 to 2 μ) sections were cut with a Porter-Blum ultramicrotome, by glass knives prepared in the laboratory. The ultrathin sections were stained in uranyl acetate for 2 hours [2] and were then examined with an RCA EMU-2D electron microscope. Thin sections were stained with azure II and methylene blue, according to the method of Richardson, Jarett and Finke,[13] and were examined with a Bausch and Lomb research microscope. Photomicrographs were taken with Kodak Contrast Process Orthochromatic cut film in an American Optical Company camera.

In the process of reducing the size of the heel wart to dimensions appropriate for electron microscopy, the operator abraded his finger. In about 4 months a wart appeared at the site of the abrasion. As will be shown, cells of each wart contained virus-like particles of similar size. These observations were considered to indicate the probability that a causative infective agent was auto-inoculated and that the agent was indeed the virus-like particles. This would be in agreement with Ciuffo's report [14] of a wart growth on his hand 5 months after self-inoculation with a wart extract, and Wile and Kingery [15] who produced warts by inoculating their subjects with a bacteriologically sterile, cell-free filtrate, and Lyell and Miles [11] who also produced warts by inoculation. Without having obtained a pure particle preparation and without having inoculated particles into the skin with the subsequent development of a wart yielding similarly infective particles, a definitive statement of the infectivity and causality of the particles cannot, of course, be made.

Observations and Discussion

A representative area of the heel wart is shown in the light micrograph of a thin (1 μ) section included for purposes of orientation (Fig. 1). Most of the cytologic and histologic features of the human wart are manifest. The lumen (L) of a blood vessel in the dermis (DE) may be seen in the lower right. An endothelial cell (E) and a red cell (C) which is passing through the endothelial lining of the blood vessel may also be distinguished. The cells, nuclei and intercellular bridges of the prickle cell layer are readily identified just above the dermis. Basal cells and a well-defined stratum granulosum do not appear in this region. The nucleus (X) of a cell in the outer area of the prickle layer may be seen. In the parakeratotic area above the stratum granulosum, nuclei (N) known to contain many of the virus-like particles are visible. Inclusion bodies (I), here disposed as nuclear caps, may also be seen. Several clusters of bacteria (B) may be recognized, but it is impossible to determine whether or not they are intracellular. (It was necessary, because of the extremely low intensity of staining of the prickle cell layer and dermis and because of the high intensity of staining of the cells in the more superficial layers, to make two separate prints of the negative, cut the regions apart and then to piece together comparably printed areas.)

The transition from the wart as manifest with the light microscope to its appearance by electron microscopy is accomplished in Figure 2,

which is a low magnification electron micrograph of an ultrathin section in a region comparable to that represented in Figure 1. The nucleus designated X corresponds to the one shown in Figure 1. The intercellular bridges or desmosomes (D) of this cell are seen much more clearly than in the light micrograph. There is margination of the nuclear chromatin although no virus-like particles are identified in the cell. This may indicate that such particles are located out of the plane of the section or that the initial infective agent was smaller than the mature particle. It is also possible that so few particles exist at this stage of infection that they are indistinguishable with the degree of magnification. Where particles are numerous, as in the nuclei (N), they may be distinguished by careful scrutiny even at this magnification. The inclusion bodies (I) are identical to those shown in Figure 1.

It should be appreciated that not every cell in a wart exhibits evidence of infection. The appearance of an apparently normal cell in the prickle cell layer of the digital wart is shown in Figure 3. The nucleus (N), mitochondria (M), rough-surfaced endoplasmic reticulum (ER), Golgi apparatus (G), desmosomes (D), nuclear envelope (NE) and plasma membrane (P) are clearly distinguishable. Many free ribonucleoprotein particles (RNP) appear in the cytoplasm. Several tufts of keratin filaments (F) are evident.

An example of one of the highly concentrated and complex fields of desmosomes encountered is shown in Figure 4. Apparently, both interdigitation and the protrusions from one cell into the indentations of another are necessary to account for the configurations seen. The clear appearance of the desmosomes indicates that the affected cells are from the prickle cell layer. In any event, the observation of several fields with concentrations of desmosomes supports Charles's contention [4] that desmosomes are developed to an abnormal extent in the malpighian cells of the wart. It was not claimed by Charles, nor can we, that these desmosomes are derived from infected cells. This is an important point, but perhaps even more important is the possibility that the presence of infected cells in the more superficial layers may stimulate the cells deep in the stratum spinosum to form a greater number of desmosomes than normal. In any event, regardless of the stimulus for their formation, an extraordinary density of desmosomes occurs in cells in the wart. It is difficult to determine the significance of Gaylord's report [3] of a failure to form intercellular bridges in affected cells as they move higher in the malpighian layer. There is little or no evidence relating to the mode or site of desmosome formation. Preliminary observations suggest that they are formed in the deeper layers of the stratum spinosum. Moreover, once formed, desmosomes persist in infected cells as long as cell boundaries

can be detected (Figs. 5 and 9 to 13). They become extremely difficult to distinguish, however, in the stratum corneum or in parakeratotic areas because keratin accumulation increases cytoplasmic density. It might be suggested that all of a cell's desmosomes are formed before it is infected and that, once formed, they persist as long as the cell can be differentiated from its neighbors. Electron microscopy supports this concept. There is no known evidence to indicate that the formation or dissolution of desmosomes are reversible. Experiments of a rather complex nature would be needed to cast further light on this situation.

Virus-like particles are evident in the nucleus shown in Figure 5, and there is a suggestion that they may also be present in the cytoplasm. A group of particles in the cytoplasm appears at the lower right. Their proximity to the nucleus and the occurrence of a definite break (double arrow) in the nuclear envelope adjacent to them suggest the possibility that they may have leaked from the nucleus. Whether such leakage should be considered an artifact or an indication of the mechanism of particle passage cannot be determined. The illustration resembles Figure 237 in Gaylord's report; [3] it was his contention that virus-like particles were developed in the nucleus and continuously leaked into the cytoplasm. This would be essentially similar to the events reported by Morgan, Howe, Rose and Moore [16] in their studies of RI-APC viruses. Actually, the nuclear membrane in Gaylord's figure 237 appears to be interrupted; thus the cytoplasmic location of the particles may here, too, be an artifact.

Marginated nuclear chromatin is quite prominent, and an arrow indicates a desmosome (Fig. 5). This clearly proves that desmosomes persist in cells which contain large numbers of virus-like particles, an observation contradicting Strauss and co-workers [7] who claimed that intercellular bridges were absent between affected cells. The margination of chromatin, characteristic of wart cells containing virus-like particles, bears a striking although apparently heretofore unnoticed resemblance to the margination of nuclear material in bacterial cells infected with bacteriophage. Mudd, Hillier, Beutner and Hartman [17] demonstrated this quite elegantly. The mechanism of the reaction is not understood; it would seem to reflect some sort of repulsion of chromatin by virus-like particles in the wart on the one hand and by bacteriophage DNA on the other.

Somewhat more convincing evidence of the cytoplasmic location of particles would be provided if a continuous nuclear envelope could be demonstrated and if the particles appeared farther from the nucleus. Both of these conditions were found to occur in a cell in a parakeratotic area very close to the surface of the wart (Fig. 6). The nucleus is

solidly packed with virus-like particles and several clumps appear in the cytoplasm. A similar distribution has been demonstrated by Lutzner,[18] who believed that they proceeded into the cytoplasm from their sites of production within the nucleus. The arrow (Fig. 6) indicates a bacterium in the cytoplasm of a parakeratotic cell. The heel wart contained a great number of bacteria, possibly representing several genera. The majority were intercellular, but many were located intracellularly as well. The fact that bacteria located within cytoplasm have been so rarely illustrated appeared to us to justify their reproduction in this report. The additional rarely made observation that many of the bacteria were in the process of division further enhanced the desirability of their illustration. For example, photomicrographs showing division of *Mycobacterium lepraemurium* in the mouse spleen and of symbiotic bacteria in roach ovary have been published by Chapman, Hanks and Wallace[19] and by Bush and Chapman.[20] The fact that the bacteria have not been optimally preserved may be attributed to the processing procedures used for tissues; these are quite different from those found effective for bacteria.[21] The essential difference is the duration of fixation; animal cells and tissues generally require 1 to 4 hours and bacteria, about 16 hours.

The most convincing evidence supporting the belief that virus-like particles do occur in the cytoplasm of wart cells was the appearance (Fig. 7) of numerous small clusters of the particles throughout the cytoplasm. Here, too, they lay in the nucleus and there was also margination of chromatin. The difference in the appearance of the chromatin in the cell of the stratum granulosum from that in the parakeratotic area was striking. The latter contained essentially no chromatin, indicating the possibility that it may have been utilized in the production of the particles.

In the absence of samples at successive intervals after inoculation with virus-like particles, it is impossible to provide a description of the sequence of events in the infection. Such a sequence might, however, follow the entrance of a small number of virus particles into each of a group of cells in an abraded area. It is uncertain whether entrance involves pinocytosis or direct contact with raw cytoplasm. In the latter circumstance the cytoplasmic membrane would presumably heal and functional integrity would be restored to the cell. Some of the particles would make their way through the cytoplasm to the nucleus, but here, again, the process of access into the nucleus is unknown. If the particles entered the cell by pinocytosis, the enclosing membranes might fuse with the outer element of the nuclear envelope, thus conveying the particles into the intracisternal space between the two membranous elements of the envelope. The particles would then have to penetrate the

inner element of the envelope. If the particles were admitted directly into raw cytoplasm or if the pinocytotic vesicle membrane was ruptured, releasing the particles into the cytoplasm, they might then enter the nucleoplasm through pores in the nuclear envelope. Once inside the nucleus, the particles would somehow influence nuclear synthetic mechanisms to produce virus-like particles. When a certain concentration of the particles is attained, they must pass into the cytoplasm, by a reversal of the nuclear entrance process or by a localized breakdown of the nuclear envelope. Their release from the cell might be via reverse pinocytosis or by cellular disintegration. The actual demonstration of these conceptual details would indeed constitute a significant contribution.

A first step in this direction was taken by Almeida, Howatson and Williams,[22] who described intranuclear particle development and related the particles to intranuclear basophilic inclusions. The particles first appeared within the nucleus in association with the nucleolus. As the amount of virus-like material increased, basophilic inclusions became more prominent; the nucleoli lost prominence and were no longer detectable in the upper region of the stratum granulosum. Our observations are in complete agreement with these. Electron microscopy has confirmed and extended Hydén's[23] and Blank, Buerk and Weidman's[24] observations. Hydén[23] studied the basophilic inclusions by ultraviolet microspectrophotometry and concluded that they represented viral substance. Blank and co-workers,[24] using light microscopy, similarly concluded that basophilic inclusions were associated with the virus-like particles. (It should be noted that it was Lipschütz[25] who first described these basophilic inclusions in warts.) It is also interesting to note that Almeida and co-workers[22] stated that the particles were rarely observed in the cytoplasm; when detected, they were considered to have been released from the nucleus by nuclear membrane disruption.

Two additional points deserve mention. First, the studies of Almeida and co-workers,[22] supporting those of Hydén[23] and Blank and co-workers,[24] tend to refute the opinions of Strauss and co-workers,[7] Lyell and Miles,[11] and Bloch and Godman[26] that eosinophilic inclusions constitute specific evidence of virus infection. Secondly, there is a marked resemblance between the process of virus formation in the human wart and that in Shope papilloma.[27] As noted by Almeida and co-workers,[22] the process in each instance involved the association of specific particles with the nucleoli of cells in the stratum spinosum, the multiplication of the particles until they occupied most of the nuclear volume in the stratum granulosum and the eventual dissolution of the affected cells.

The appearance of cells at the junction of the stratum granulosum and zone of parakeratosis is of considerable interest. At the lower right

corner of Figure 8 several dense keratohyalin granules appear in a cell of the stratum granulosum. Just above these granules a nucleus contains many virus-like particles. At this magnification, desmosomes between two cells are detectable (arrows), again indicating that desmosomes persist in cells containing virus-like particles. The keratohyalin granules to the left and above the nucleus are of particular interest for they appear to be fusing. This interpretation is supported by the fact that keratohyalin masses in cells more superficially located become larger. Lyell and Miles[11] have made similar observations. It should also be noted that the more superficial keratohyalin has reduced density. In the most superficial area (Fig. 14) cytoplasm of a cornified cell is replaced by a moderately dense, amorphous substance apparently derived from fused or coalesced, and probably chemically modified, keratohyalin granules. Several nuclei of cells in the parakeratotic area contain virus-like particles in the top and left of Figure 8. Prominent marginated chromatin and large keratohyalin masses are noteworthy. Figures 9 and 10 are higher magnifications, exhibiting greater detail. In each, several persisting desmosomes are indicated by arrows.

The nuclei and portions of two cells in the parakeratotic area are shown in Figure 11. The degree to which nucleoplasm may be replaced by virus-like particles, the diminution in the amount of chromatin and the persistence of desmosomes (arrow) in these heavily infected cells are clearly shown. A higher magnification (Fig. 12) shows a desmosome in greater detail (arrow). How the accumulation of keratohyalin material may obscure desmosomes is readily seen.

In addition to illustrating the nature of chromatin margination, Figure 13 exhibits the degree to which virus-like particles may replace the nucleoplasm. Also evident are the persistence of desmosomes (arrow), a keratohyalin granule (double arrow), the apparent coalescence of keratin fibrils presumably comprised of tonofilaments, and an intranuclear inclusion identical in density and texture to the large keratohyalin cytoplasmic granule to the right. The relationship between the fibrils and the dense granule resembles that illustrated by Zelickson.[28] It was his interpretation that the dense keratohyalin acted as a matrix or cementing substance which ensheathed the tonofilaments in the course of keratinization. This is essentially in agreement with our interpretation, but of course normal keratinization is not observed in the wart.

The dense intranuclear inclusion provokes several questions. First, is it truly intranuclear or does it merely lie in an indentation in the nuclear envelope? Second, is the inclusion actually a nucleolus? It seems likely that it is truly intranuclear since virus-like particles are located too near the inclusion to permit the intervention of a nuclear envelope. Identical density and texture in the intranuclear and the cytoplasmic

inclusions and the fact that neither has the granular character of a nucleolus suggest that the inclusion is not a nucleolus. It may be classed as a Cowdry Type B nuclear inclusion.[11] The cytoplasmic inclusion, on the other hand, is probably an enlarged, bizarre keratohyalin granule, as suggested by Blank and Rake.[29]

According to Gaylord,[3] the linear arrays of virus-like particles (Fig. 14) appear as the number of particles increases. It is apparent, however (Figs. 6 and 11), that this is not necessarily the case. Several bacteria may also be seen in the cytoplasm of cells in the parakeratotic area (Fig. 14).

As indicated above, electron micrographs of bacterial cells in infected tissues, in various stages of division, have been rarely published. Figures 15 to 22 constitute such a group. The cell wall (W), plasma membrane (M) and low density nuclear area (N) are shown in Figure 17. The empty spaces may represent artifacts, probably sites previously occupied by lipid dissolved in tissue preparation. On the other hand, they may have the location of a material of different nature from the cytoplasm gouged out in the sectioning. The process of division in the micro-organisms seems to be characterized by a simultaneous and centripetal deposition of plasma membrane and cell wall. This differs from the process described in *Bacillus cereus* by Chapman and Hillier; [21] in this organism only the cell wall participated; a plasma membrane was not demonstrated. An unidentified micro-organism described by Chapman [30] also differed in that division was characterized by the formation of a nearly complete membrane septum before centripetal deposition of a cell wall was effected. Stages in division prior to the completion of the membrane septum and cross wall are shown in Figures 15 to 19. A completed septum and cell wall are shown in Figures 20 to 22. The dense particles in Figures 19 and 21 are, from their size, probably ribonucleoprotein. The occurrence of both a bacterial cell and virus-like particles in the same cytoplasmic area is shown in Figure 20.

Summary

Electron microscopy of ultrathin sections of human warts from the heel and finger exhibited virus-like particles in both the nucleus and cytoplasm. Extraordinarily dense aggregates of desmosomes were associated with the cells, and these persisted as long as the cells could be distinguished. The virus-like particles had a maximum diameter of approximately 38 mμ. Nonviral intranuclear inclusions were identified and were probably distinct from the nucleolus. A series of micrographs illustrated the mode of cellular division in bacteria occurring as secondary invaders of the lesion under study.

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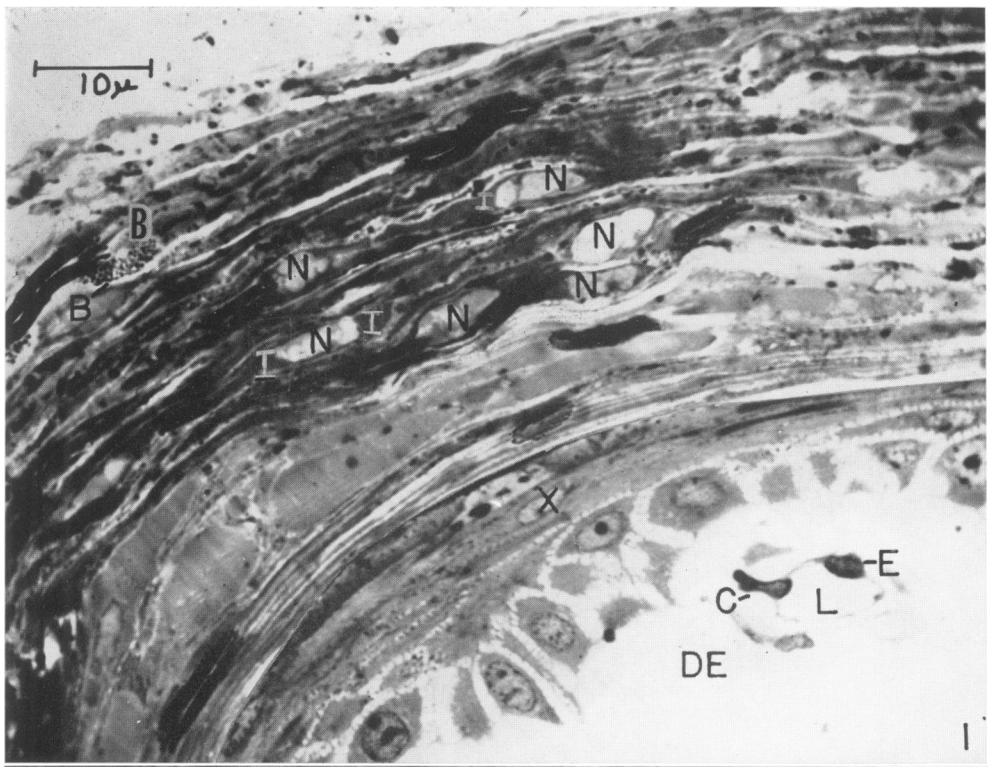
[*Illustrations follow*]

Legends for Figures

Unless otherwise stated, the magnification symbol in each figure indicates 1 μ.

Fig. 1. A survey photomicrograph of a heel wart cut at 1 μ. Shown are the lumen (L) of a blood vessel in the dermis (DE), an endothelial cell (E), a red cell (C), a nucleus (X) in the outer prickle cell layer, nuclei (N) containing virus-like particles, inclusion bodies (I) and bacteria (B). × 1,500.

Fig. 2. An electron micrograph of a region comparable to that shown in Figure 1. The nucleus (X) corresponds to that similarly designated in Figure 1. Desmosomes (D) are clearly seen. Nuclei (N) contain visible virus-like particles and inclusion bodies (I). Both are comparable to those shown in Figure 1. × 8,000.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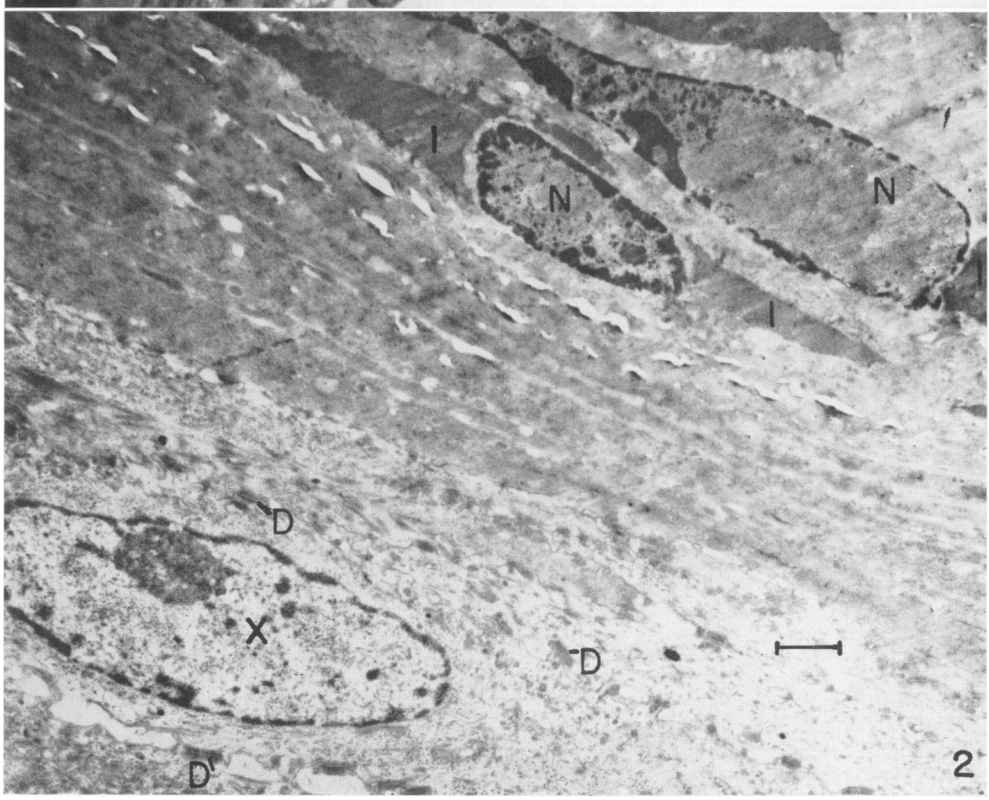
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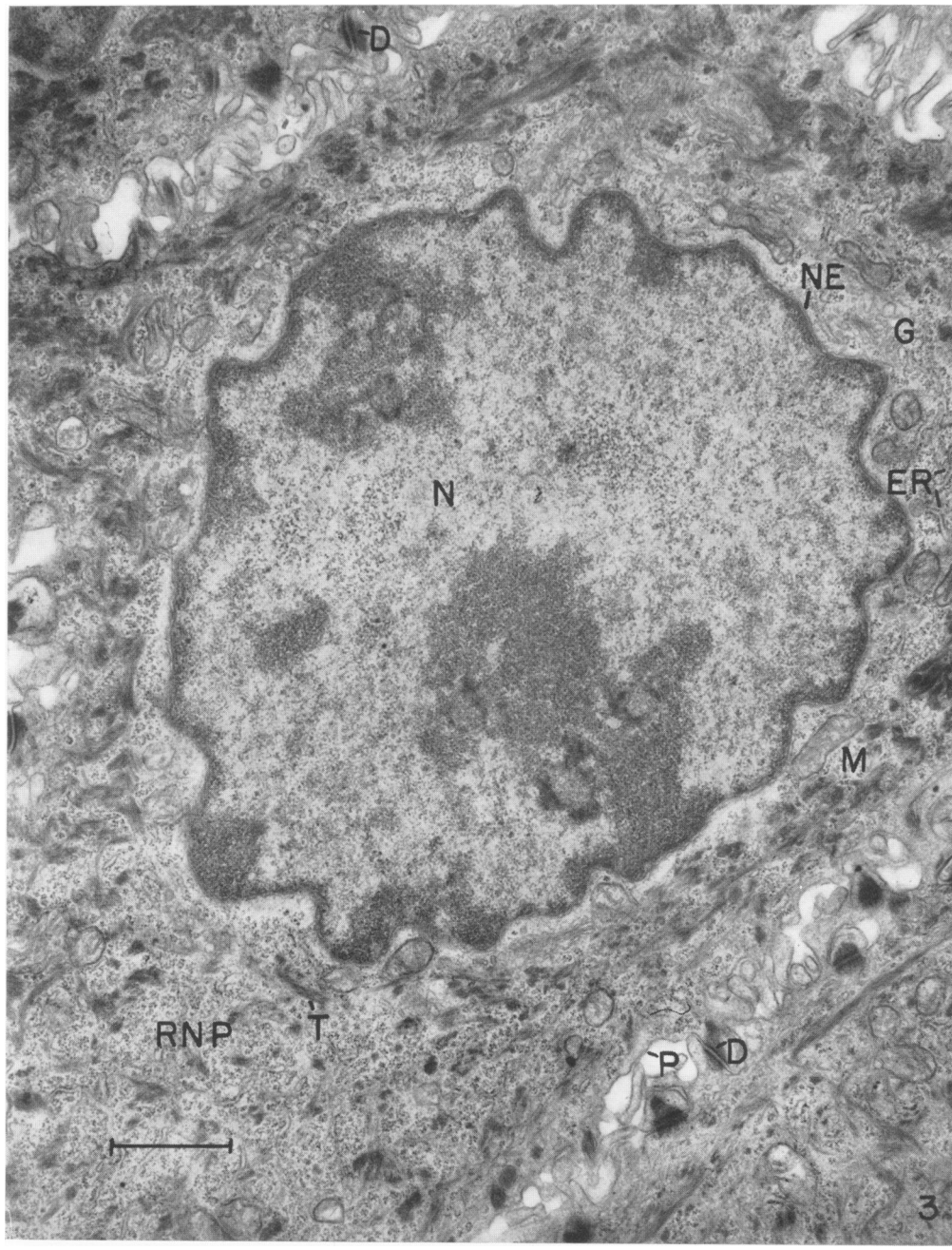


FIG. 3. A cell in the prickle cell layer in the finger wart is apparently normal. Nucleus, N; mitochondria, M; rough-surfaced endoplasmic reticulum, ER; Golgi material, G; desmosomes, D; nuclear envelope, NE; plasma membrane, P; free ribonucleoprotein particles, RNP; tufts of keratin tonofilaments, T. × 16,000.

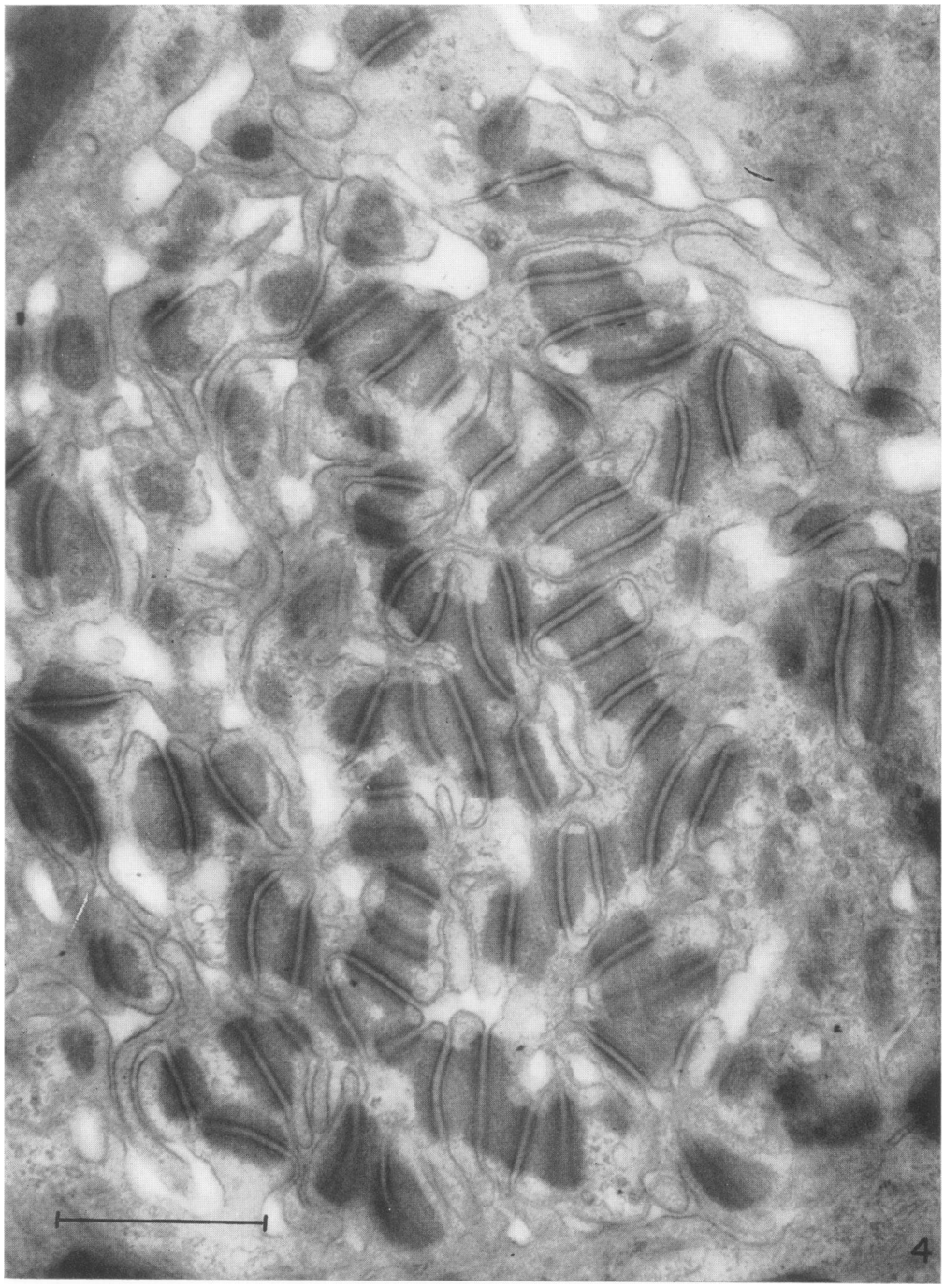


FIG. 4. The fields of desmosomes have become strikingly concentrated. × 29,000.

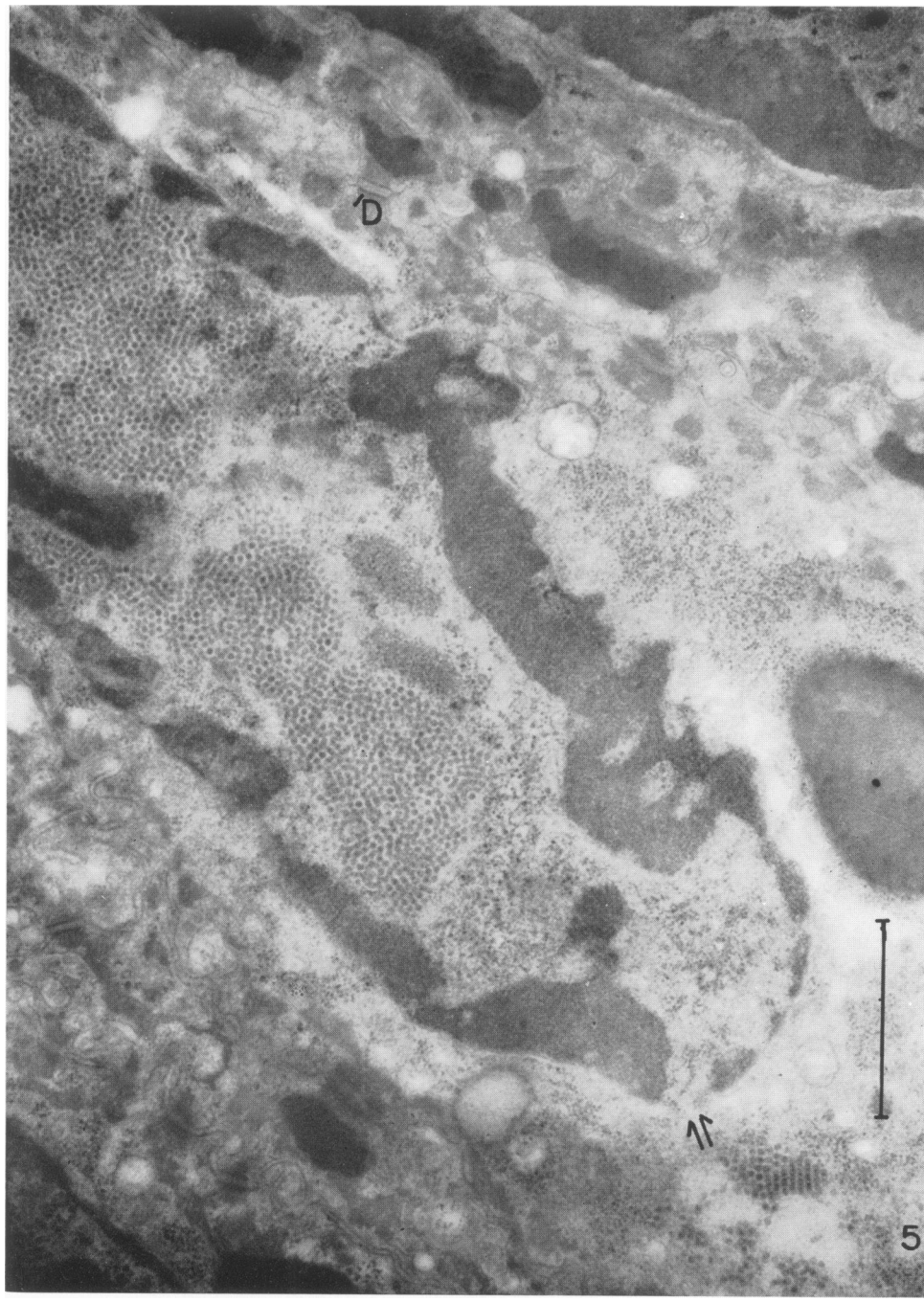


FIG. 5. Virus-like particles appear in both the nucleus and cytoplasm. In the latter, the possibility of leakage due to artifact exists. A discontinuity (double arrow) in the nuclear envelope supports this possibility. Desmosome, D. × 27,000.

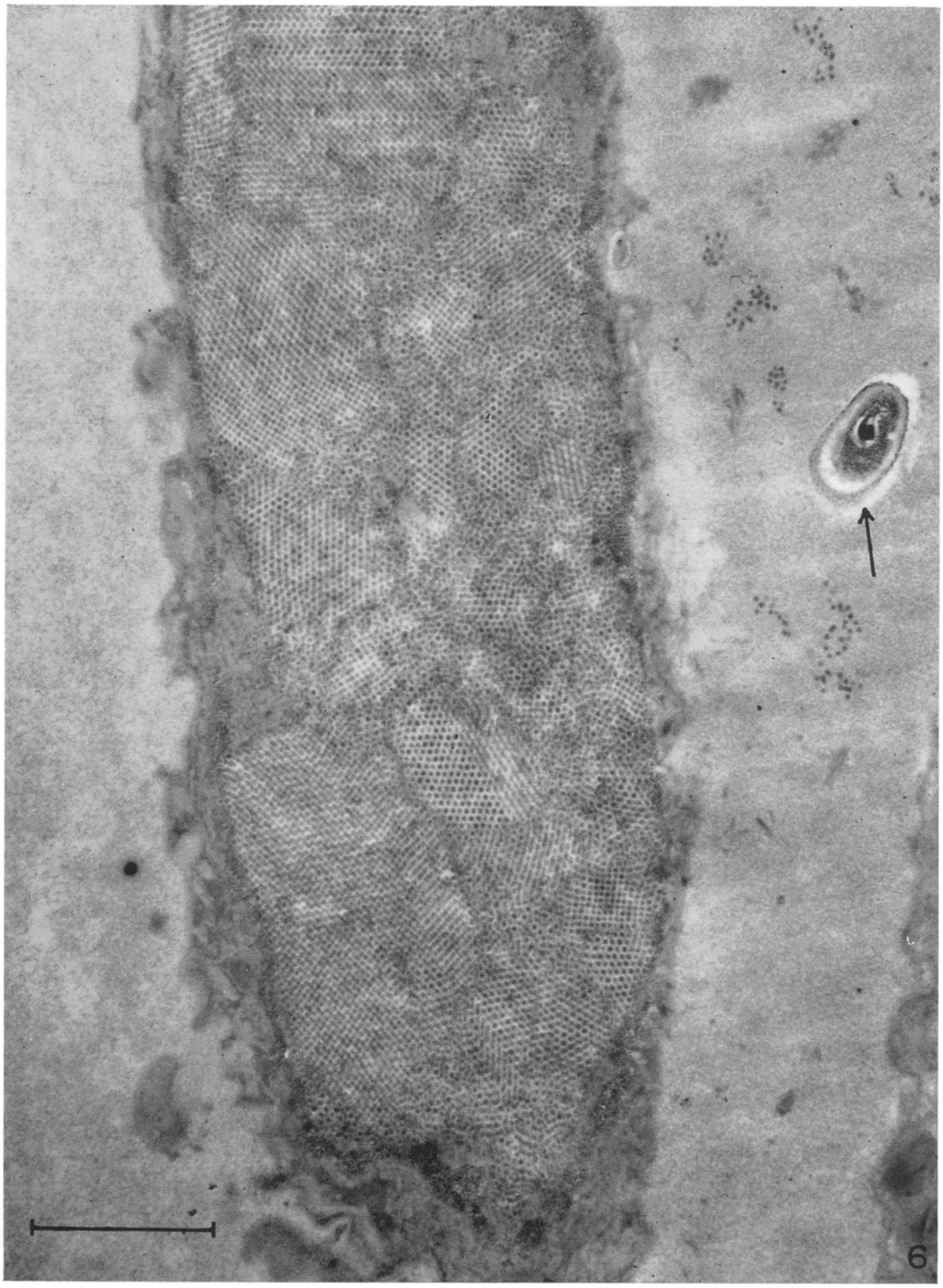


FIG. 6. Intracytoplasmic virus-like particles are less likely to be the result of artifact than those shown in Figure 5. The arrow indicates a bacterial cell. × 25,000.

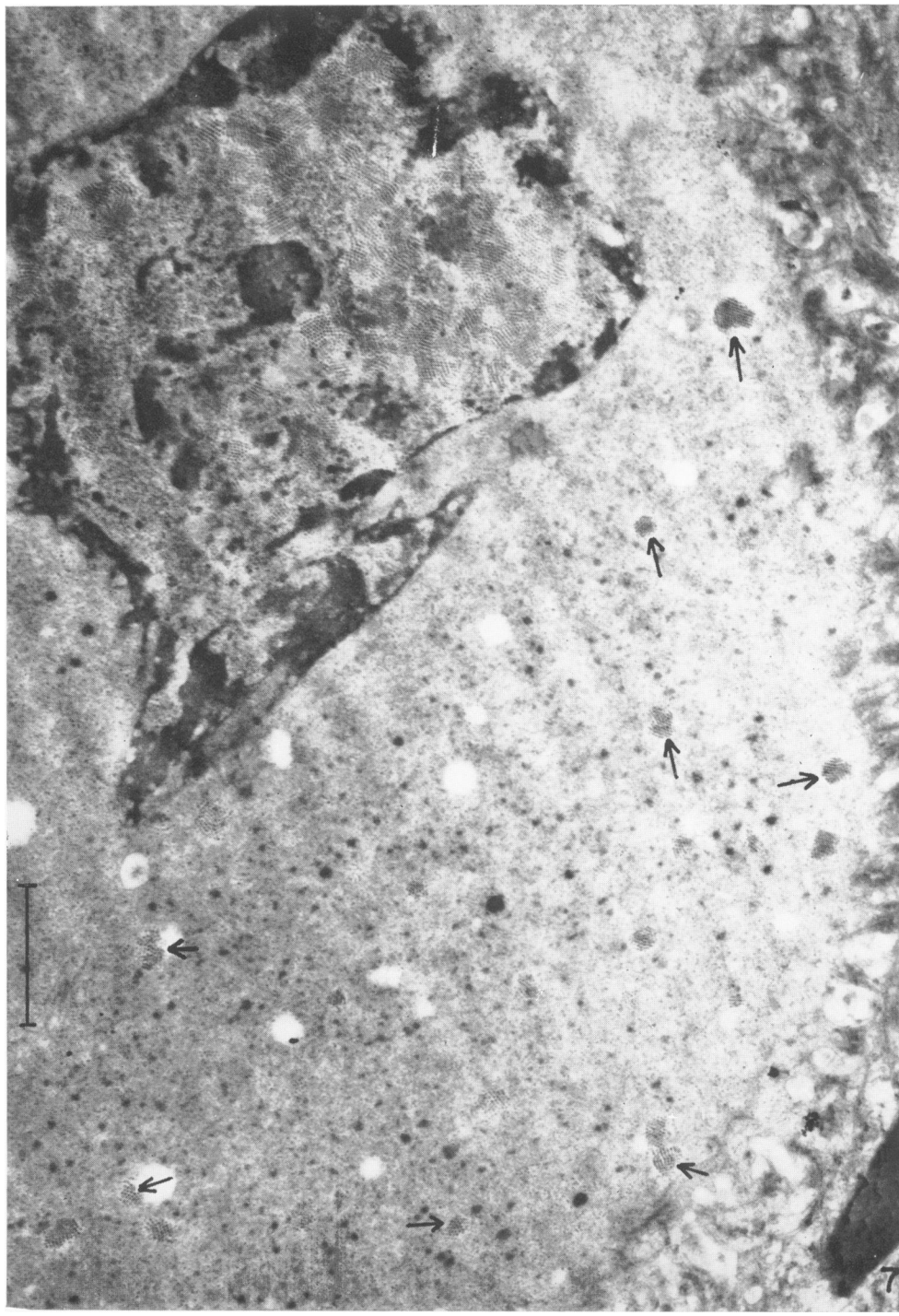


FIG. 7. Clusters (arrows) of virus-like particles are widely scattered in the cytoplasm. This constitutes convincing evidence that such a particle disposition is not an artifact. × 10,000.

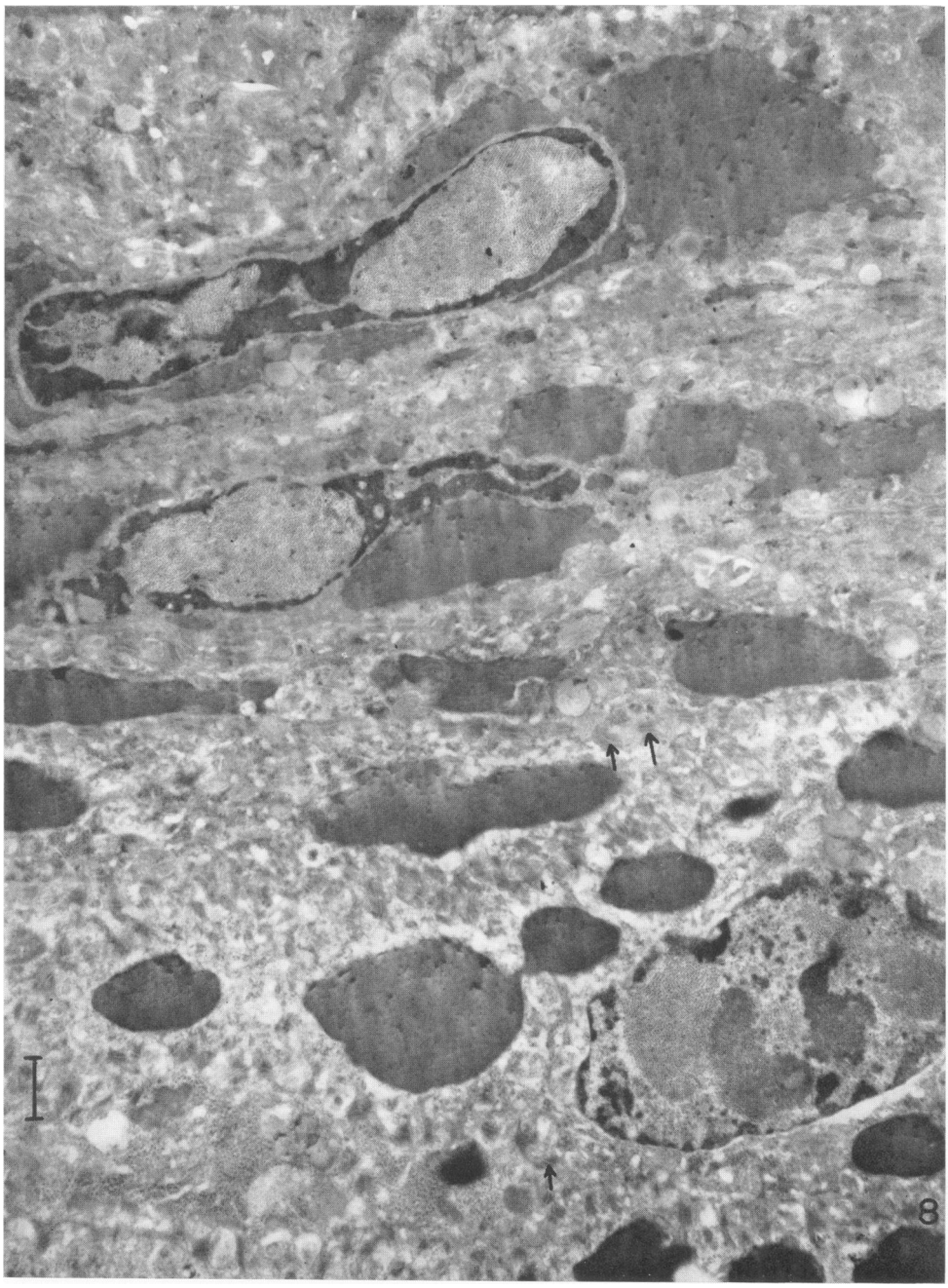
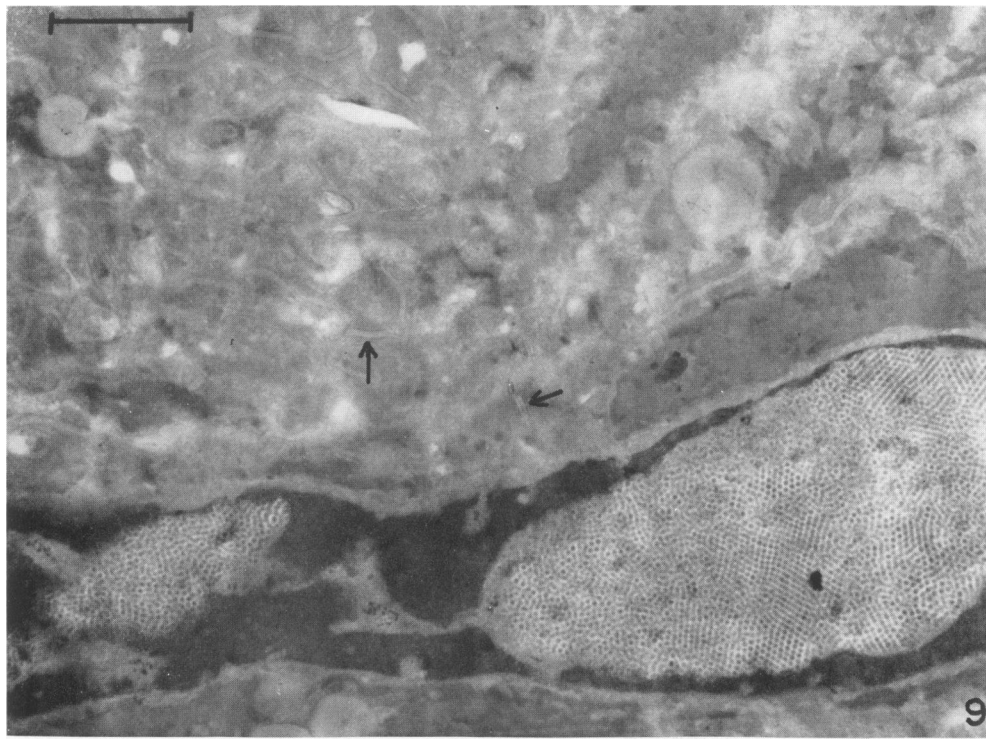


FIG. 8. A junction of stratum granulosum and a zone of parakeratosis. Desmosomes (arrows) are just visible. × 8,000.



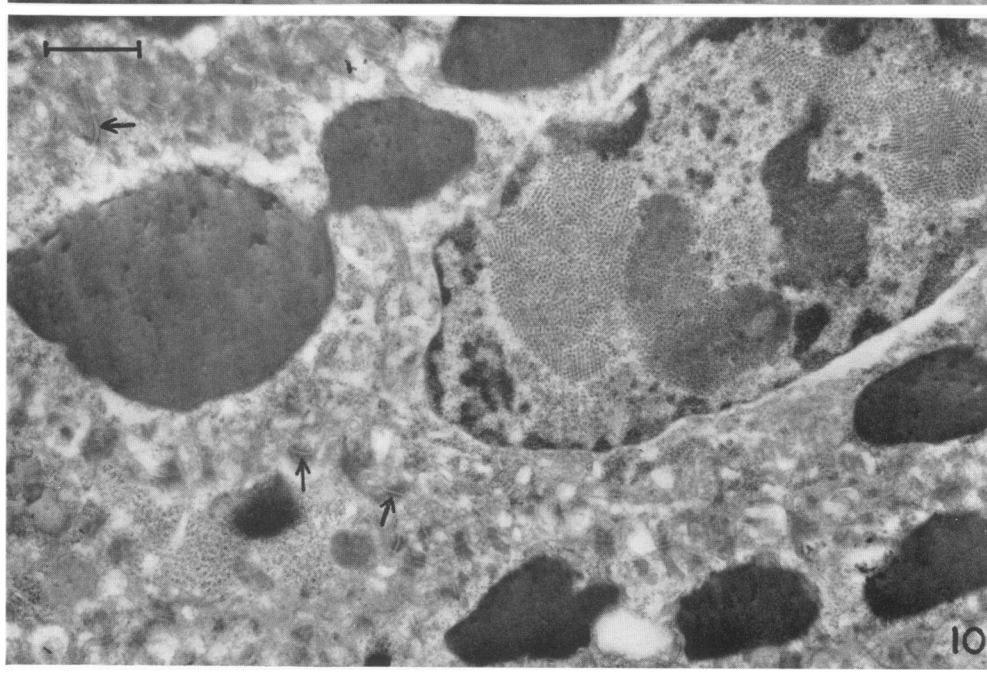


FIG. 9. A higher magnification of an area in Figure 8. Several of the persisting desmosomes are indicated by arrows. × 18,000.

FIG. 10. Same as Figure 9. × 12,000.

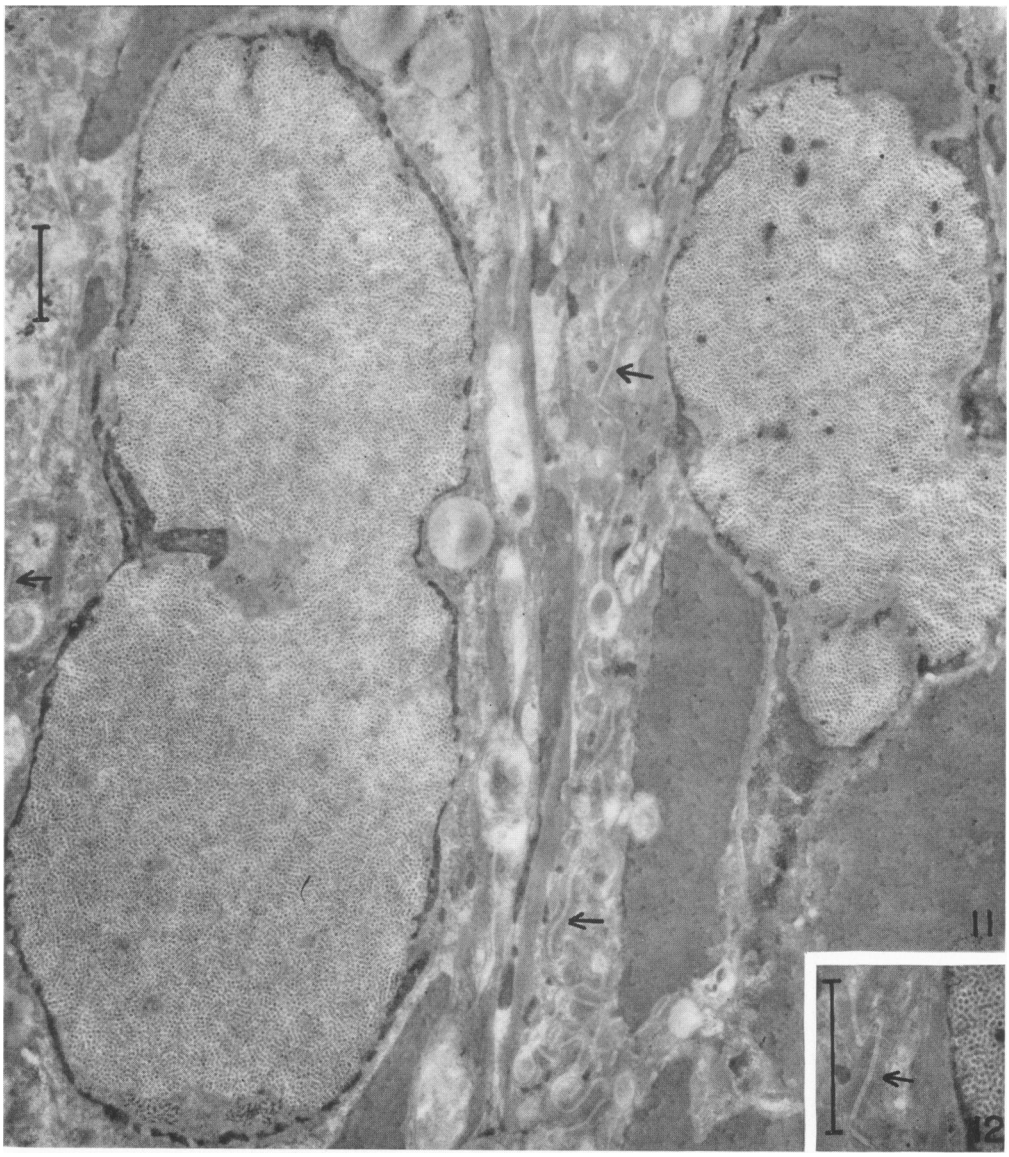


FIG. 11. The nuclei and portions of two cells in a zone of parakeratosis. Packing of the nuclei with virus-like particles is striking. There is reduction in the amount of marginated chromatin. Desmosomes (arrows) are manifest. × 12,000.

FIG. 12. A higher magnification of a portion of Figure 11. A persistent desmosome is indicated by an arrow. × 20,000.

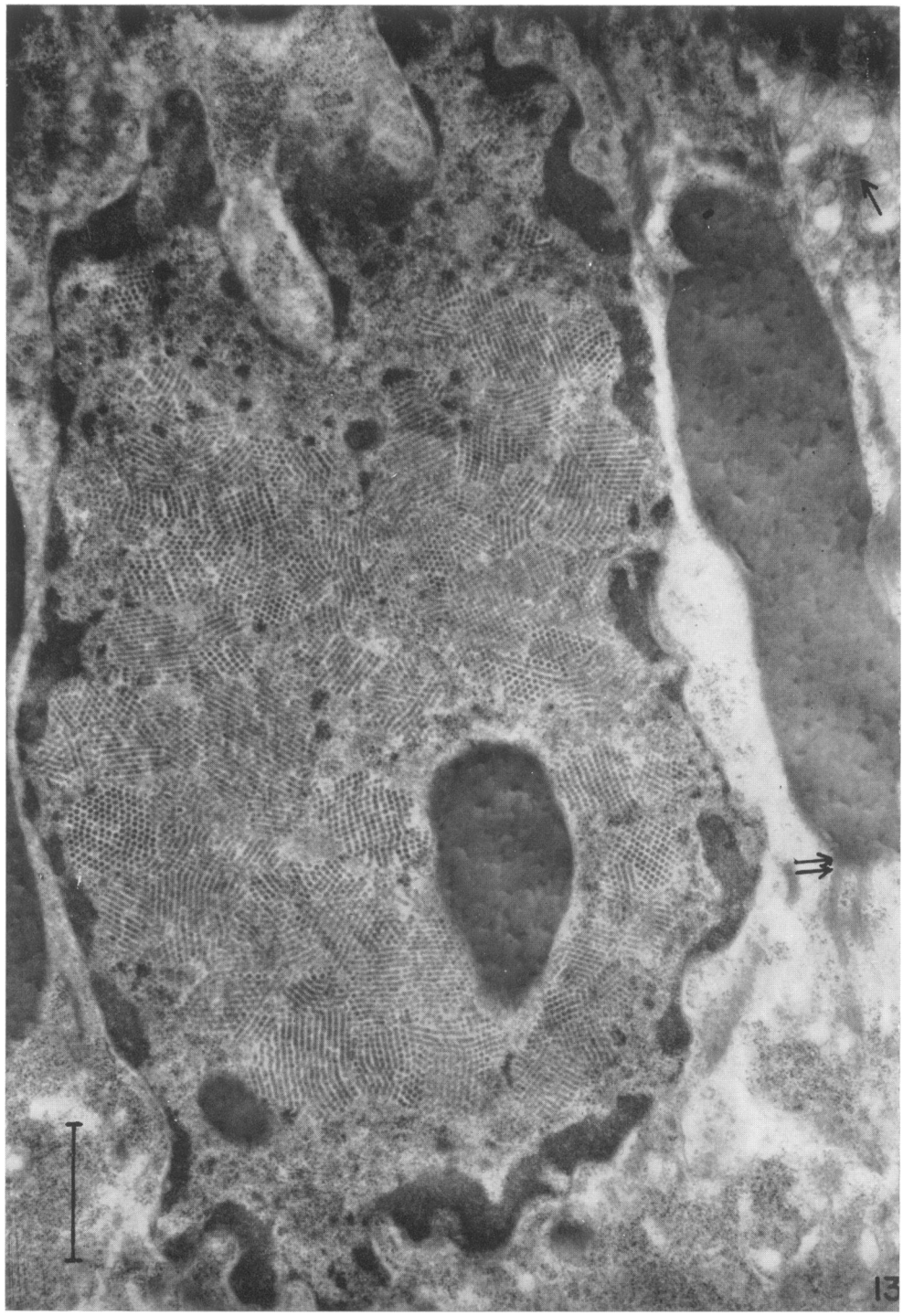


FIG. 13. In addition to persistence of a desmosome (arrow) in an infected cell, there is also apparent coalescence of a group of keratin fibrils and a keratohyalin granule (double arrow). × 20,000.

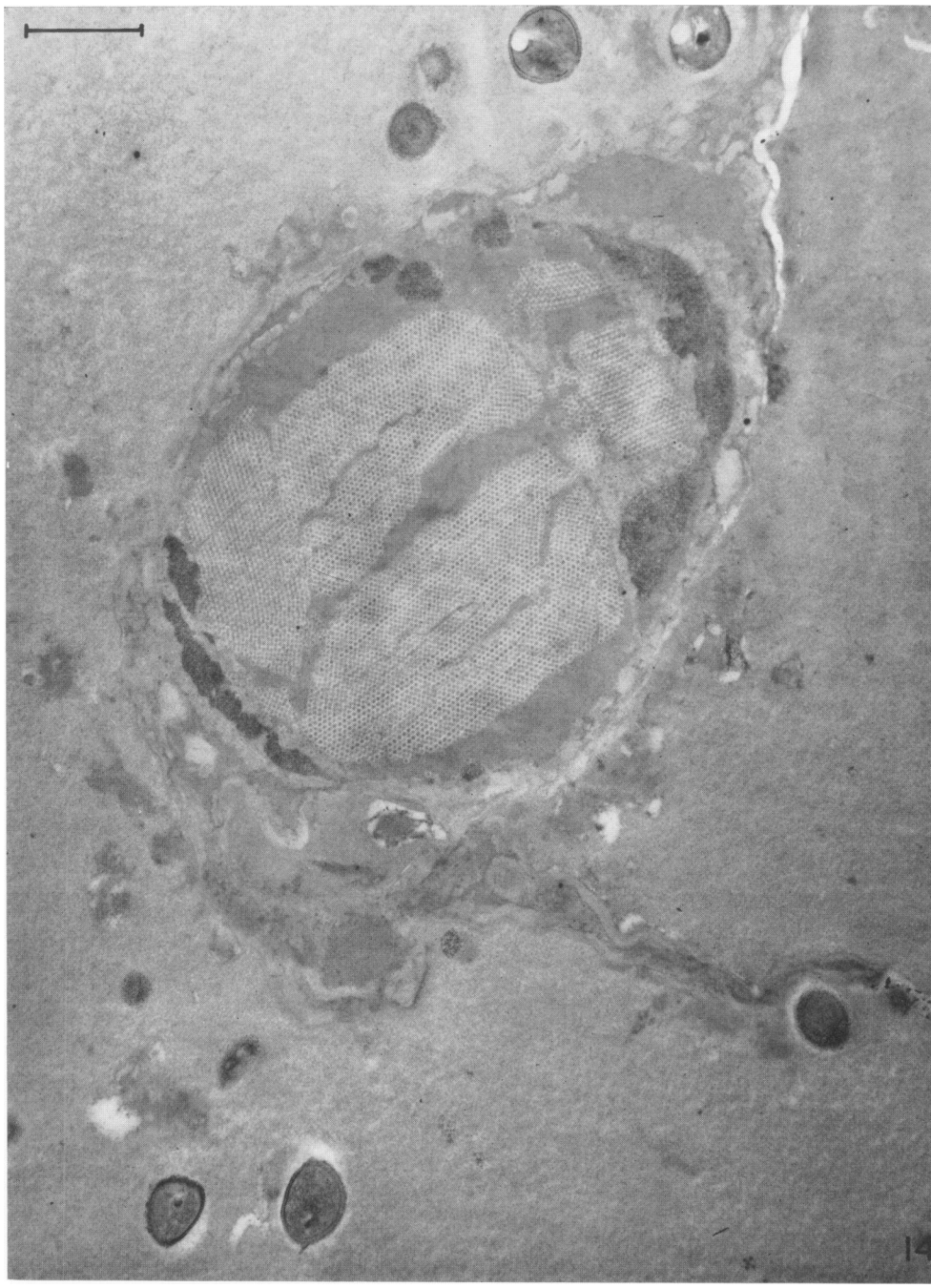
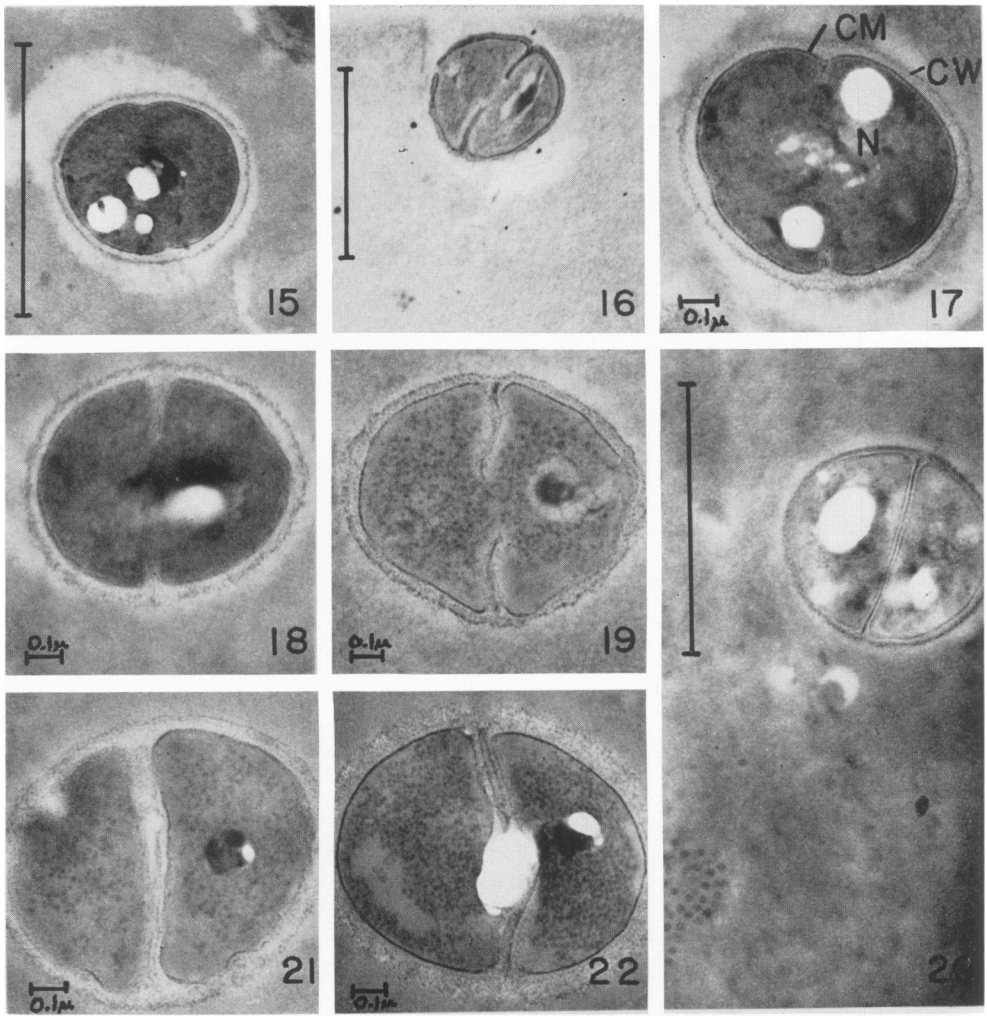


FIG. 14. Linear arrays of virus-like particles are quite prominent. Several bacterial cells are also shown. × 16.000.



FIGS. 15 to 22. Various stages of cellular division of bacteria. In Figure 17 the various structural features of the bacterial cells are labeled. Cell wall, CW; plasma membrane, CM; low density nuclear area, N. Figures 15 to 19 illustrate stages in division before completion of membrane septation and cross wall formation. Figures 20 to 22 reveal the appearance of completed cross wall and membrane septum. Fig. 15: × 35,000. Fig. 16: × 25,000. Fig. 17: × 49,000. Fig. 18: × 49,000. Fig. 19: × 49,000. Fig. 20: × 35,000. Fig. 21: × 49,000. Fig. 22: × 49,000.