

ELECTRON MICROSCOPE OBSERVATIONS ON INTRACELLULAR
VIRUS-LIKE PARTICLES ASSOCIATED WITH THE CELLS OF
THE LUCKÉ RENAL ADENOCARCINOMA*

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Relatively few malignant neoplasms have been described in amphibia. The one that has been studied most extensively is the Lucké renal adenocarcinoma of the frog, *Rana pipiens*. This malignant tumor occurs in from two to four per cent of frogs of this species, collected in the Lake Champlain region of northern Vermont and adjoining areas of Quebec. In a series of papers Lucké described its histopathology (17), reported the occurrence of distant metastases (18) and demonstrated the presence in some of the tumors of intranuclear inclusion bodies of a kind commonly seen in virus diseases (17, 22). It was subsequently shown by him that tumors could be induced in frogs of the susceptible race by injection of desiccated or glycerinated preparations of tumor. Similar inoculation of other races or species was without effect. These observations led Lucké to conclude that the tumors were probably caused by a species-specific, organ-specific agent having the characteristics of a virus (19, 20). Additional supporting evidence for this conclusion was provided in later work of Schlumberger and Lucké (31) in which the tumor was carried for many generations by serial intraocular transplantation. In the course of these experiments it was noted that frogs bearing intraocular tumors very often developed kidney tumors. Similar results have been reported by Rose and Rose (30) in anterior chamber transplants in adult frogs, and by Briggs (4) in tadpoles with tumor implants in the tail fin. In both instances persistently growing adenocarcinomas frequently developed in the kidneys even though the implanted tumor tissue itself regressed. The most reasonable interpretation of these findings seems to be that a tumor agent passed from the implanted tissue to the kidney and there induced a tumor of the same kind. Further evidence of the viral nature of the tumor-inducing material has been provided by the work of Duryee and Doherty (8, 9) who have recently established its filtrability.

In the present investigation, thin sections of frog renal tumors have been

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examined with the electron microscope. In *some* specimens the cells were found to contain virus-like particles of uniform size and distinctive form, which may be the postulated tumor agent. Sundry observations have been made on the distribution of these particles within the cells, on their internal structure and their mode of development.

Materials and Methods

The light microscope observations presented here are based upon the examination of twelve spontaneous tumors from specimens of *Rana pipiens* obtained from a dealer in the Lake Champlain region of Vermont. Four of these tumors were also studied with the electron microscope. For light microscopy, tumor tissue was fixed in Bouin's or Zenker's fluids, or in formol-sublimate. After paraffin embedding, sections were cut at 5μ and stained with hematoxylin and eosin or by the Feulgen reaction counterstained with light green. For electron microscopy, small blocks of tumor, $1 \times 1 \times 2$ mm., were fixed in 1 per cent osmium tetroxide, adjusted to pH 7.6 with veronal-acetate buffer (28). After fixation for 2 to $2\frac{1}{2}$ hours, the tissue was dehydrated in graded concentrations of ethyl alcohol and infiltrated for a total of 3 hours in three changes of *n*-butyl methacrylate monomer, containing 2 per cent luperco¹ as a catalyst. Polymerization was promoted by exposure to mild heat (45–50 C.) in an oven for 24 hours. Sections 20 to 30 $m\mu$ in thickness were cut on a mechanically advanced thin-sectioning microtome of the type designed by Porter and Blum (29). The sections were examined directly without removing the plastic embedding medium.

Electron micrographs were made with an R.C.A. electron microscope, model EMU-2E with a 30μ aperture in the objective pole-piece. The exposures were made at original magnifications of 2,000 to 8,000 diameters and the images were enlarged photographically to the desired size. The magnifications were determined by calibration of the microscope with a replica of a diffraction grating having 30,000 lines to the inch.

OBSERVATIONS

This study of the frog renal adenocarcinoma is an outgrowth of an earlier electron microscope investigation of the fine structure of the normal frog kidney. Our previous detailed analysis of the fine structure of the normal kidney provides a sound basis for comparison in the following discussion of the cytopathology of the Lucké renal tumor. At present the results of the earlier study are available only in abstract (11) but inasmuch as they are soon to be published in full, no space will be devoted here to description or illustration of the normal kidney.

Histological Appearance of the Tumors.—The tumors are typical adenocarcinomas comprised of sheets or solid cords of epithelial cells delimiting irregular spaces (Figs. 1 and 2). The spaces are often filled with eosinophilic masses of degenerating cells and cellular debris. The epithelium is composed of tall columnar cells, one or two layers deep, supported by a sparse connective tissue stroma. In some areas, a narrow, discontinuous striated border is discernible on the free surface of the epithelium while, in other areas, no such surface speciali-

¹Luperco CDB, a preparation of 2,4-dichlorobenzoyl peroxide manufactured by the Novadel-Agene Corporation, Buffalo, New York.

zation is visible. The cytoplasm of the tumor cells is finely granular and somewhat more basophilic than that of normal renal epithelium. The ovoid nucleus usually has a single large nucleolus and numerous small clumps of chromatin randomly distributed in the karyoplasm (Fig. 4).

In some tumors, a considerable number of the nuclei are swollen and contain prominent inclusions that stain faintly with eosin (Figs. 3 and 5). In the abnormal nuclei, the inclusion body is centrally located, irregular in outline, and often surrounded by a clear zone. The nucleolus is displaced to one side and the chromatin is in coarse masses closely applied to the nuclear membrane. Some nuclei exhibit "margination of the chromatin" without a definite inclusion body being discernible, others show advanced nuclear disorganization and beginning degeneration. Such profoundly disturbed cells may be found in close proximity to others whose nuclei appear perfectly healthy. The cytoplasm of cells with nuclear abnormalities often contains angular elongated bodies that differ from the nuclear inclusions in that they stain lightly with hematoxylin. Cytoplasmic inclusions seem to have been overlooked in Lucké's original accounts of the histopathology of this tumor (17, 21), but they have been described by Duryee (9) and are of considerable interest in relation to certain of the electron microscope observations that will be described below.

It should be emphasized at the outset that inclusion bodies are not found in all of the tumors examined. In Lucké's large series they were observed in approximately 50 per cent of the tumors, but in our experience to date they have been noticed in only about 30 per cent. The majority of the electron microscope observations presented here were made on four tumors which did show abundant nuclear inclusions in stained sections examined with the light microscope.

In these tumors there were marked local variations in the incidence of nuclear abnormalities. Within the same area, cells with healthy appearing nuclei, and cells with well developed nuclear and cytoplasmic inclusions can often be found in close proximity to one another. In the interests of clarity the fine structure of the two categories of cells will be described separately.

The Fine Structure of Tumor Cells Lacking Inclusion Bodies.—The free surfaces of cells bounding the spaces within the tumor are often covered with slender microvilli that account for the appearance of striated borders in sections observed with the light microscope. These slender processes are very numerous on some cells and sparse on others. They are never as uniform in length or as closely packed and orderly in their arrangement as are the microvilli which form the brush border of the normal frog nephron (Figs. 11 and 12). Nevertheless, they are sufficiently well developed to suggest that the tumor originates in the proximal rather than the distal segment of the nephron. The contour of the contact surfaces of adjacent tumor cells and the surface specializations found at the cell base are also consistent with this interpretation. The lateral

surfaces of the tumor cells, like those of normal cells in the proximal tubule, are smooth throughout most of their extent but show a few shallow interdigitations near the basement membrane. Irregularly spaced along these surfaces are local thickenings of the opposing cell membranes which resemble those observed with the electron microscope at the so called "desmosomes" or "intercellular bridges" in various normal epithelia (12). These seem to be more numerous and better developed in the tumors than in normal renal epithelium.

The cell base is irregular in contour and shows numerous infoldings of the plasma membrane that produce a compartmentation of the basal cytoplasm similar to that seen in cells of the proximal segment of the normal nephron.

The cytoplasmic matrix of the tumor cells is of rather low density. The endoplasmic reticulum, which is not particularly well developed, is usually represented by circular or oval profiles that correspond to transverse or oblique sections through a continuous system of membrane-bounded tubules that run a sinuous course through the cytoplasm. Occasionally however there are longer profiles that consist of parallel membranes, continuous with one another at the ends. These appear to represent sections of large, flattened vesicles or cisternae. A moderate number of minute granules of ribonucleoprotein are adherent to the membranes of the reticulum but an even greater number are evenly distributed through the cytoplasm giving it a finely stippled appearance (Figs. 19-21). The dispersion of the ribonucleoprotein granules throughout the cytoplasm is doubtless responsible for the diffuse cytoplasmic basophilia observed in these cells with the light microscope.

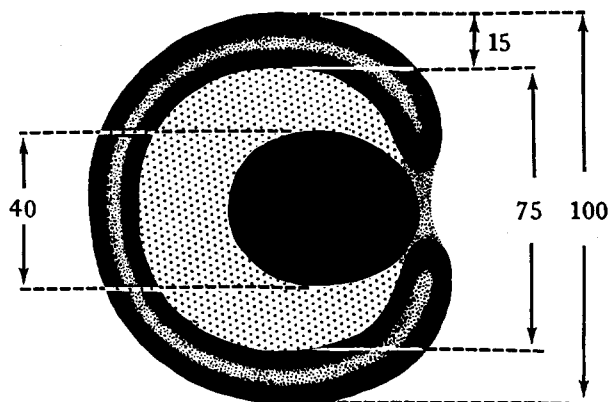
Mitochondria occur in all regions of the cell but are particularly numerous in the basal portion. They tend to be smaller than usual, but otherwise do not differ significantly from mitochondria of normal kidney. The membranous septa or cristae that project into their interior are present in normal abundance as are also the coarse granules found in the mitochondrial matrix between cristae (Figs. 19 and 22).

The Golgi complex has the same basic structure as that of normal cells, being made up of aggregations of small vesicles and parallel arrays of smooth membranes organized into two or more distinct groups that are usually located in the supranuclear region.

The nucleus is ovoid and bounded by two membranes about 20 $m\mu$ apart. The innermost of these represents the nuclear membrane proper, and the outer is regarded as the limiting membrane of the cytoplasm. The karyoplasm consists of a matrix of low density containing fine granules of three or more kinds that differ in size and density and in their state of aggregation. The prominent nucleolus is made up of closely packed granules of very uniform size (10 to 15 $m\mu$) and of considerable density. Scattered through the karyoplasm are large irregularly shaped masses of fine granules of somewhat lower density than those comprising the nucleolus (Figs. 6 and 8). These granular masses of intermediate

density correspond in their shape and distribution to the clumps of chromatin in Feulgen-stained nuclei observed with the light microscope. In addition to the delicate desoxyribonucleoprotein granules of the chromatin, the background nucleoplasm is irregularly stippled with dense granules that are distinctly larger and more variable in size and shape. These often form conspicuous clumps 100 to 150 $m\mu$ in diameter randomly scattered in the nucleus (Fig. 8). Their chemical nature and their relationship to the other granular components of the karyoplasm is unknown.

It may be said in summary, that carcinoma cells conforming to the foregoing description show no presently recognizable stigmata of virus infection and exhibit no single distinctive structural feature that could be regarded as pathognomonic of neoplastic cells. They differ from normal cells of the proximal con-



TEXT-FIG. 1. A diagrammatic representation of the virus particle found in the Lucké renal adenocarcinoma. The approximate dimensions are given in millimicra.

voluted tubule principally in their tall columnar form, the absence or imperfect development of the brush border, the abundance of their cytoplasmic ribonucleoprotein granules and the small size of their mitochondria.

Fine Structure of Tumor Cells Containing Nuclear and Cytoplasmic Inclusions.—Mingling with tumor cells of the type just described are others that are basically similar but contain several cytoplasmic and karyoplasmic components that have not been observed in normal cells and are believed to be related to virus infection. The commonest of these abnormal structures is a particle of uniform size and distinctive form found in considerable numbers in the cytoplasm and occasionally in the nucleus as well (Figs. 10, 19, 20). These particles are spheroidal in shape and 95 to 110 $m\mu$ in diameter. They consist of a dense capsule about 15 $m\mu$ thick enclosing a clear central zone 75 $m\mu$ in diameter which is of very low density giving the appearance of a central cavity. Within this "cavity" is a round, dense body 50 $m\mu$ in diameter (Text-fig. 1). In some

planes of section the inner body or granule is completely surrounded by a clear zone and thus appears to be central but when cut in other planes, it is clear that the inner body is eccentrically placed and is fixed on one side to the inner aspect of the capsule. The capsule is appreciably thinner at this point than elsewhere and in some instances appears to have a circular opening that is occluded by the inner body. Studied at high magnification the capsule is found to be a double-walled structure comprising outer and inner dense layers approximately 5μ in thickness and separated by a 5μ intermediate zone of lower density.

Because of the striking resemblance of the particles described here to particles found by Morgan and his collaborators in cells known to contain virus of herpes simplex (25) we take the liberty of referring hereafter to these encapsulated spherical bodies as "virus particles" realizing that their viral nature is not established by such morphological resemblances alone.² They are uniform in size and appearance except for occasional anomalous particles that have a second dense capsule (Fig. 20) outside of the usual one. As a rule they are individually and randomly distributed in the cytoplasm but sometimes they occur in large clusters enclosed by a thin membrane (Fig. 10). The virus particles show no constant relationship to the endoplasmic reticulum or Golgi complex and rarely if ever invade mitochondria. In some cells they congregate immediately beneath the cell surface and, in such instances, extracellular particles are also found occupying the interstices between the microvilli or lying free in the closed alveolar spaces within the tumor (Figs. 11 and 12). The occurrence of considerable numbers of these distinctive particles outside of the cells is one of the principal reasons for believing that they represent the mature infectious form of the virus. The extracellular particles have, in addition to their own capsule, a thin loosely fitting membrane that is not present on intracellular particles. From time to time two are found enclosed by the same membranous investment. Such extracapsular membranes seem to be detached portions of the plasma membrane of the host cell and apparently are acquired by the virus particles in their passage through the cell surface. In Fig. 12 two virus particles are seen in the expanded tip of a microvillus which is constricted below them in a manner suggesting that the portion of the villus containing the particles might soon have been cast off into the lumen, had not the activity of the cell surface been arrested by the fixative.

² Proof that the various particles that have been described in electron micrographs of virus infected cells are actually the virus is still lacking. In recognition of this uncertainty it has become customary to refer to newly discovered bodies of this sort as "virus-like" particles. Actually, little is gained in accuracy by saying that they are "like" other particles found in virus infected cells when the viral nature of those other particles has not been definitely established. "Particles-presumed-to-be-virus" would be a more conservative term but this expression is all too cumbersome to use repeatedly. We have decided therefore, in the interests of simplicity and readability to omit the qualifying phrases and to refer simply to the "virus particles." It is hoped that the reader will understand that we hold the same reservations about their identification as others who use the term "virus-like particles."

Tumor cells containing virus particles often have one or more conspicuous bundles of coarse filaments in their cytoplasm. These are about $25\text{ m}\mu$ in thickness, 4 or $5\ \mu$ in length, and generally more or less parallel in their arrangement (Figs. 19, 21, 23). In high resolution micrographs the coarse filaments are seen to be made up of finer filamentous subunits estimated to be from 5 to $8\text{ m}\mu$ in thickness. The origin of the filaments is unknown. Mature virus particles are often found in close relation to these bundles of dense filaments and in one or two high resolution micrographs a few fine filaments could be traced to the inner body of one of the associated virus particles (Fig. 19), but the significance of this observation is not clear.

Such sheaves or bundles of dense filaments are never seen in normal kidney cells or in the tumor cells that show no morphological evidence of virus infection. They are quite common, however, in the cytoplasm of tumor cells that have nuclear inclusions. There they form sizeable structures that are certainly large enough to be visible with the light microscope. It is probable that they correspond to certain of the elongated cytoplasmic inclusions seen in preparations stained with hematoxylin (Fig. 3).

A third type of abnormal structure found in the cytoplasm of virus containing carcinoma cells consists of an aggregation of membrane-limited vacuoles each containing numerous minute vesicles of rather uniform size (Figs. 21–23). An homogeneous, dense (? osmiophilic) substance occupies the interstices between the larger vacuoles (Fig. 22). A few virus particles are occasionally found within the vacuoles, but there is no indication that they are formed there and their incorporation within these peculiar vacuolar inclusions appears to be accidental. To date it has not been possible to assemble from the electron micrographs a sequence of formative stages which would provide a clue to the origin of these inclusions. It is conceivable that the vacuoles are degenerating mitochondria and that the small vesicles in their interior are formed in the disintegration of the cristae mitochondriales. An alternative possibility is that the vacuolar inclusions represent a gross abnormality of the Golgi complex. However, the coexistence in these same cells, of Golgi material of essentially normal configuration, argues against this. Neither of these interpretations accounts for the network of homogeneous dense material. It is clear that the observations to date are an inadequate basis for a definitive interpretation of the origin and significance of these structures but it is our impression that they have nothing directly to do with virus reproduction but are the product of a characteristic pathological reaction of the host cell to the presence of the virus.

The nuclear "chromatin" of tumor cells that are free of virus particles, is composed of fine granules aggregated into irregularly shaped masses of varying size and indefinite outline (Fig. 8) and these occur in all parts of the nucleus. On the other hand in carcinoma cells that do contain virus particles, the granules of the chromatin are more closely packed and seem to be completely segregated from other granular material of the karyoplasm. The chromatin masses thus

appear denser and have more sharply defined borders. In such cells the nucleus often contains a large central mass of irregular outline that appears to correspond to one of the nuclear inclusion bodies seen with the light microscope (Figs. 6 and 7). In the presence of such a body, the chromatin masses and nucleolus are displaced to the periphery of the nucleus. The small clumps of dense granules that are observed in limited numbers in the karyoplasm of apparently virus-free tumor cells are still more abundant in nuclei of cells containing virus particles (compare Figs. 6 and 7 with Fig. 8).

The objects that seem to be nuclear "inclusion bodies" consist of large numbers of thin walled, spherical vesicles embedded in a fine, granular, or filamentous matrix. The vesicles are of uniform size with a smooth limiting membrane and an amorphous content of very low density. They are usually packed loosely and randomly in the inclusion bodies but in some instances they are precisely aligned in straight parallel rows (Fig. 7) resembling the crystalline arrays of particles described in the inclusions of certain other types of animal viruses (27). In vesicles that chance to be sectioned in the right plane, a hiatus is visible in the wall. This does not seem to be an artifactual discontinuity, but a naturally occurring opening in the membrane (Figs. 15 and 17). Although the majority of the vesicles comprising an inclusion appear quite empty, a few contain an elliptical or spherical granule of about the same size and density as the inner body of the cytoplasmic virus particles already described. In some instances the granule is centrally placed within the vesicle, in other instances it is neither entirely within nor without, but is lodged in the opening of the incomplete membrane (Figs. 9 and 15). Similar dense bodies are also found free in the karyoplasm in close proximity to clusters of empty vesicles (Figs. 14, 17, 18). It is tempting to interpret these images as stages in a developmental process whereby a dense body or granule is formed in the karyoplasm and subsequently enters a preformed membranous envelope to give rise to an immature virus particle. No internal structure has been resolved in these dense bodies but some show a slender filament or tail-like process extending from one end (Figs. 15 and 16). Such filamentous projections are more commonly seen on those bodies that are free in the karyoplasm or on those partially within vesicles than they are on those that are entirely enclosed by a membrane (Fig. 18).

The origin of the inner bodies of the immature virus particles is not clear. There is no evidence that they arise from the large masses of marginated chromatin as suggested by Morgan in the case of herpes virus (25). It appears more likely that they are formed by condensation of the granular or filamentous material that occurs in small dense clumps scattered throughout the central area of the nucleus (Fig. 7).

In addition to the masses of thin walled vesicles that make up the bulk of the nuclear inclusions there are occasional aggregations of vesicular structures of slightly smaller size that have two thin membranes (Figs. 9 and 13).

These have no inner dense body and their relation to the other vesicular structures in the karyoplasm and to the development of the virus particles is obscure.

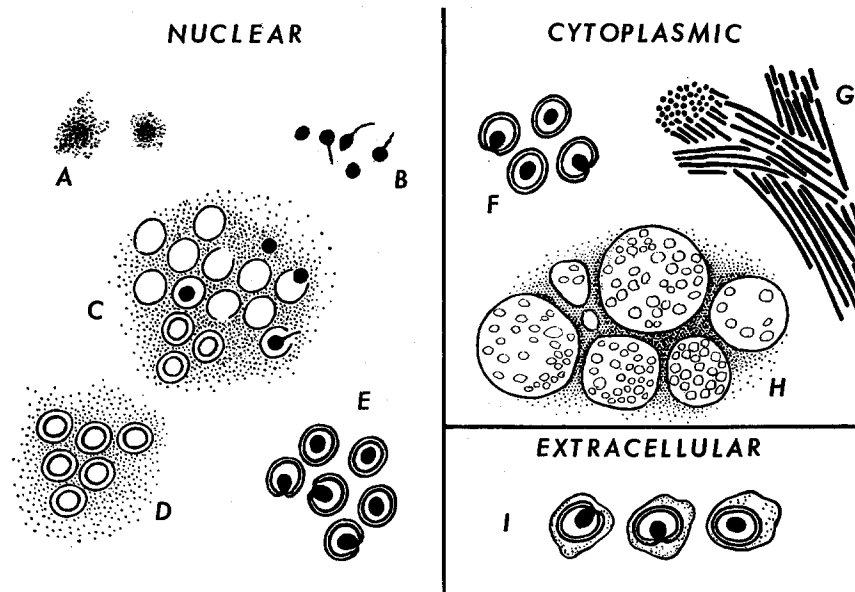
Typical mature virus particles that are identical to those in the cytoplasm are sometimes encountered in the nucleus. They do not occur individually as they do in the cytoplasm but are generally closely associated in large clusters and enclosed by a thin membrane (Fig. 9). Such membrane-bounded aggregations of particles are often situated near the nuclear membrane. It will be recalled that similar collections of virus particles enveloped in a delicate membrane occur occasionally in the cytoplasm also (Fig. 10) and it is easy to speculate that these arise in the nucleus and pass out into the cytoplasm where the surrounding membrane undergoes dissolution and liberates the particles.

DISCUSSION

The electron micrographs obtained in the present study provide the first visual demonstration of particles in the tumor cells that might be the postulated tumor agent. Because of their great morphological similarity to the particles observed by Morgan *et al.* in cells experimentally infected with herpes simplex and other viruses, it is easy to conclude that the particles found in the Lucké tumor are virus particles of some kind. The possibility cannot be excluded, however, that they may be a virus that is unrelated to the causation of the tumors. The characteristic particles are not found in all of the tumors examined and when they are present they are not seen in all of the cells. When studied in histological sections, the kidney tumors fall into two categories, those in which conspicuous nuclear inclusions are present and those in which they are lacking. Our experience to date seems to warrant the generalization that when typical inclusions are seen in a tumor with the light microscope, virus particles will be found in electron micrographs of the same tumor. In the absence of nuclear inclusions the particles are either entirely lacking or they are present in such small numbers as to escape detection by the inadequate tissue sampling afforded by electron microscopy.

Although fully cognizant of the uncertainties involved in any effort to reconstruct a dynamic process from a limited number of static images, we submit, in resumé, the following tentative interpretation of the intracellular formation of virus particles in the Lucké tumor. At multiple foci within the nucleus, numerous smooth contoured spherical vesicles 75 to 80 $m\mu$ in diameter are formed (Text-fig. 2 C). Their limiting membrane is often incomplete on one side and their content is amorphous and of very low density so that they appear empty. These vesicles are closely aggregated and embedded in a fine granular matrix having a texture somewhat different from the rest of the nucleoplasm. When the vesicles and their associated matrix accumulate in large masses they form the nuclear inclusion bodies that are visible with the light microscope. Among the vesicles are found a few dense, spheroidal bodies 30 to 40 $m\mu$ in

diameter that sometimes show a slender tail-like process projecting from one side (Text-fig. 2 B). These seem to arise by condensation of granules or filaments of macromolecular dimensions that occur in clusters scattered through the matrix (Text-fig. 2 A). Each of these bodies enters one of the open vesicles to give rise to an immature virus particle consisting of the dense body ($40 \text{ m}\mu$)



TEXT-FIG. 2. Diagram presenting the nuclear, cytoplasmic, and extracellular structures that occur in the renal tumors, but are not found in normal frog kidney. In the nucleus there are: A, small dense aggregations of fine granules or filaments; B, dense spheroidal bodies 30 to $40 \text{ m}\mu$ in diameter sometimes having a slender tail-like projection on one side; C, clusters of membranes forming hollow spheres or vesicles of uniform size that are embedded in a moderately dense granular matrix, some of the vesicles containing a dense inner body; D, spherical particles with double membranes but no inner granule; and E, mature virus particles with a dense spheroidal inner body enclosed in a double membrane. In the cytoplasm there are: F, mature virus particles; G, bundles of coarse, dense filaments; and H, inclusions made up of aggregations of vacuoles filled with minute vesicles. In the extracellular spaces there are: I, virus particles that are enclosed in a thin, ill fitting outer membrane not found on intracellular virus particles.

enclosed in a single membranous envelope ($80 \text{ m}\mu$). In further development a second enclosing membrane is formed, resulting in a particle with a spheroidal inner body, eccentrically situated within a double-membraned capsule (Text-fig. 2 E). This is believed to be the mature, infectious stage of the virus. In this form the particles are released into the cytoplasm, either individually or in clusters contained within a thin membrane. They ultimately pass from the cytoplasm through the free surface of the cell and into the extracellular spaces

of the tumor. In leaving the host cell the particles acquire an additional covering which appears to be a detached portion of the cell membrane (Text-fig. 2 I).

This highly speculative scheme of intracellular virus development agrees, in the main, with the hypotheses put forward by Morgan *et al.* (25), and Bernhard *et al.* (1, 2), to account for the forms which they have encountered in electron micrographs of other types of virus-infected cells. In the case of herpes simplex, Morgan envisions that a primary body (30 to 40 $m\mu$) is first formed from viral matrix in the nucleus and a single membrane is deposited around it. On passing into the cytoplasm the particle is believed to acquire a second membrane. From a study of electron micrographs of the cytoplasmic inclusions in the Shope fibroma, Bernhard *et al.* were led to believe that membranes developed within a mass of homogeneous, finely granular "viroplasm," each membrane delimiting a portion of the matrix which ultimately condenses to form the dark center of a virus particle. Thus the suggestion offered here that the inner, dense body of the Lucké tumor virus and its investing membranes arise separately in the nucleoplasm and are subsequently combined to form an immature virus particle differs somewhat from the mechanisms previously proposed for other animal viruses. It is no more remarkable, however, than the sequence of events that is postulated for reproduction of bacterial viruses. It is generally accepted for bacteriophage that the protein envelope of the infective particle does not enter the bacterial host. In the subsequent reproduction of the virus, the coats investing the progeny are therefore believed to be synthesized entirely from protein of the host and recent evidence points to a two-stage process whereby synthesis of nucleic acid and protein envelopes goes on *independently* until the stage of maturation at which time the two components are combined into infective particles by a highly specialized assembly mechanism that is as yet poorly understood (22, 23).

In his classical paper on the nuclear changes associated with virus infections, Cowdry (5, 6) defines two morphologically distinct types of intranuclear inclusion bodies. Those inclusions that are centrally placed in an enlarged nucleus that shows distinct "margination" of the chromatin, were designated type A. They are characterized by acidophilic staining and a negative Feulgen reaction. The changes in the karyoplasm are progressive, leading ultimately to disintegration of the nucleus. The type B inclusions, on the other hand, consist of relatively small acidophilic granules or droplets that are localized in certain areas of the nucleus. The chromatin pattern is not notably altered and the abnormal process does not lead to degeneration of the nucleus. Both types of inclusion generally appear amorphous with the light microscope and their nature and significance have long been a subject of controversy. They have been regarded by some investigators as a complex of virus particles and nuclear material. Others have considered them to represent a stage in the non-specific degeneration of the host cell nucleus. A majority seem to have favored the

latter interpretation, because the apparent absence of Feulgen reactive material in type A inclusions casts serious doubt upon the thesis that the inclusions are largely composed of the virus particles themselves. The present study sheds further light upon the nature of one type of nuclear inclusion. The acidophilic masses in the nuclei of the Lucké tumor are typical examples of Cowdry's type A inclusions. Electron micrographs reveal that they contain relatively few complete virus particles but instead are largely composed of hollow, spherical vesicles of the same size as the virus. A few of these contain dense inner bodies and are considered to be immature virus particles but the vast majority of the vesicles appear to be quite empty. The large inclusions visible with the light microscope thus seem to be the result of a great overproduction of the viral membranes. The matrix itself does not appear to be rich in nucleic acids. If the essential nucleoprotein component of the virus particle resides in its dense inner body, the very small number of these present in the inclusions could well account for the absence of a detectable Feulgen reaction in most of the inclusions. There is no ready explanation, however, for the elaboration of such an excessive number of empty vesicles as a byproduct of virus multiplication. This does not seem to be confined to the Lucké virus. In an electron microscope study of the intracellular forms of the viruses of vaccinia, ectromelia, and molluscum contagiosum, Gaylord and Melnick (13, 14) noted the occurrence of numerous complete membranes forming hollow spheres that were apparently empty. Bernhard also reported that it was not uncommon in the cytoplasmic inclusion bodies of the Shope fibroma to find complete membranes forming spheres that were seemingly devoid of contents. To account for this it was suggested that such empty vesicles may be the result of a disturbance in the normal sequence of events whereby the viral membranes are formed before the diffuse matrix appears. The significance of these apparently empty spheres remains unexplained, but this much can now be said of the type A inclusions of the Lucké tumor, they do not seem to be non-specific degenerative changes in the karyoplasm, but are made up of many empty viral membranes embedded in a moderately dense granular matrix. They contain relatively few complete virus particles.

The observation that the extracellular form of the virus is enclosed in a thin extracapsular membrane is of interest in relation to the mechanism of virus release. In some of the electron micrographs the relation of certain of the particles to the cell surface indicates that the thin outer layer on the extracellular particles is probably a portion of the host cell membrane that is contributed to the virus as it emerges from the cell. Also in mammary tumors of mice, Bernhard (2) observed virus-like particles that were located in the tips of cell processes projecting into intercellular spaces, and he described the extracellular forms of the virus as having a loose outer membrane that was not present on the intracellular particles. These observations have a bearing upon the question of

how virus is released from infected cells. It is commonly thought that liberation of virus always involves lysis of the cell surface. The lysis may either be generalized, resulting in complete disruption of the host cell, or localized to a single cell process that contains an accumulation of virus particles (7). The present findings suggest that virus release may not always depend upon lysis but may involve the budding off from the cell surface of minute vesicles containing an individual virus particle or sometimes more than one. Such a mechanism is compatible with continued survival of the virus-releasing cell. Furthermore, if the virus is cast off in an envelope that is essentially a small detached portion of the host cell this could have interesting implications for the survival of the virus in the "extracellular" environment. In recent *in vitro* experiments on the reaggregation of dissociated tissues it has been shown that the selective cohesion of like cells is mediated by specific forces of attraction residing in the cell membranes (32, 33). Reaggregation fails in the presence of antibody against that particular cell type (32). If one considers these interesting experiments in relation to the method of virus release just described, they provide an interesting basis for speculation as to the mechanism whereby an organ-specific virus becomes attached to cells of the appropriate type.

At present it is impossible to fit all of the abnormal structures observed in the tumor cells into a single logical scheme of virus development. For example, the foregoing account of the interrelation of the various structures found in the nucleoplasm, omits from consideration those clusters of vesicles that have a double membrane but which lack an inner body (Text-fig. 2 C and D). Since the number of thin walled vesicles formed, is greatly in excess of the number of dense inner bodies available for virus formation, it is conceivable that these double-membraned spheres represent abortive particles which have gone on to form a second concentric membrane without first acquiring a central body.

Likewise no satisfactory explanation can be offered for the irregular bundles of coarse, dense filaments found in the cytoplasm of the tumor cells (Text-fig. 2 G). Filaments of this kind have not been described as a non-specific reaction of cells to injury and neither have they been found in other non-virus tumors. Hence it is believed that they are related in some way to the presence of the virus but just how is certainly not clear. No such structures have been reported in electron micrographs of cells experimentally infected with viruses of herpes simplex (25), fowlpox (26), vaccinia, or molluscum contagiosum (24). In the Shope fibroma Bernhard *et al.* (1) found parallel arrays of thin lamellae that were closely associated with cytoplasmic masses of virus or virus precursors. These were referred to as "crystalloids" and were tentatively interpreted as byproducts of "viral metabolism." In the Lucké tumor, however, the arrangement of the filaments within the sheaves or bundles is not sufficiently well ordered for them to be described as "crystalloids" and it is doubtful whether they bear any relation to the lamellar structures found in the Shope fibroma.

It seems more likely that they are analogous to the filamentous forms that have been described for the viruses of influenza or of Newcastle disease. In the case of these two viruses the significance of the filaments is not certain. They are dismissed by some workers as a pathological product of cells damaged by virus, but others consider them to be a stage in virus reproduction. In the present instance, typical virus particles are often found in close proximity to the bundles of filaments but the two are so different in their morphology that it is difficult to see how one could be derived from the other. Yet, one is led to this conclusion unless he prefers to believe that the tumor cells are harboring more than one virus. In an earlier study of the fine structure of the normal frog kidney (11) no filaments of this kind were observed.

There seems to be no reason to assign a role in virus multiplication to the large "vacuolar inclusions" in the cytoplasm (Text-fig. 2 H). It is more likely that they result from degenerative changes in cell organelles or that they are a byproduct of the defensive reaction of the cell to invasion by the virus. Their component vacuoles, both large and small, could easily arise from the membrane systems of the mitochondria, endoplasmic reticulum, or Golgi complex, but the source of the dense amorphous material that binds them together is less easily explained.

The bundles of filaments and the vacuolar inclusions are both large enough to be visible with the light microscope but Lucké, who first described the intranuclear inclusions in the tumor cells, either failed to observe the associated cytoplasmic inclusions or attached no significance to them. Duryee (9) has recently described irregular acidophilic bodies within cytoplasmic vacuoles of Lucké tumor cells in tissue culture. These he interprets as the characteristic "inclusion bodies" of the tumor. He notes also that the nucleoli are large and hyperactive and that they sometimes approach the nuclear membrane and appear to discharge material into the cytoplasm where it is believed to accumulate to form an "inclusion body." From his studies of living renal carcinoma cells *in vitro*, Duryee concludes that the tumor is essentially a virus disease of the nucleolus (8, 9). This interesting thesis is not substantiated by the electron micrographs of the present study. Although the nucleoli are indeed large, we attribute no special significance to this finding, because nucleolar enlargement is a common feature of malignant cells. No evidence of extrusion of nucleolar material has been found in the electron micrographs and there is nothing in the fine structure of the nucleolus to indicate that it is specifically affected by the virus.

It seems likely that some confusion may result from inconsistencies in the terminology used by different workers. The intranuclear inclusion bodies first reported by Lucké and described in greater detail here, are apparently quite different from the cytoplasmic masses which Duryee now describes as the "inclusion bodies." The former are typical, type A inclusions such as are very

commonly found in virus diseases and seldom elsewhere. The latter are not peculiar to the tumor cells but, according to Duryee, also are found in limited numbers in rapidly growing cultures of normal lung, bladder, and kidney. Hence, in our opinion, they cannot be regarded as "inclusion bodies" in the specific sense of that term as it is commonly used in describing the cytopathology of virus diseases. While the observation of living cells in tissue culture has a number of obvious advantages over the study of static images in photomicrographs or electron micrographs it also introduces certain difficulties of interpretation. For example, vacuolization of the nucleolus, hypertrophy of the centrosphere, and accumulation of refractile granules in the cytoplasm are common events in tissue cultures of various cell types but these changes are relatively uncommon in the same cell types *in vivo*. Thus, in tissue cultures of renal adenocarcinomata it may be difficult to ascertain which activities of the cells are related to the presence of virus and which are attributable to the abnormal environment of the culture vessel.

There is as yet no definitive proof that the virus particles described here are the causative agent of the tumors but it does not seem unreasonable to believe that they may be. If this assumption is made, the problem of masked or latent virus immediately arises out of the necessity for explaining the fact that nearly two-thirds of the tumors show no inclusion bodies with the light microscope and no recognizable virus in electron micrographs. Are the cells of such tumors actually free of virus or is it present in a latent form that at present cannot be identified morphologically? It is generally accepted that virus can persist in a lysogenic strain of colon bacillus through many generations without visible expression, but during this time, it retains the potentiality of producing infectious particles and of destroying its host, when reactivated (23, 16, 3). Some viruses that produce cancer in animals can likewise exist in a latent condition. For example, fowl tumors have been observed which initially yielded virus, then failed to for some time, and later yielded virus again (15). The possibility cannot be denied that the virus associated with the Lucké tumor may be able to exist in a symbiotic relationship with the host cells for long periods without producing particles of distinctive morphology that are easily recognizable as virus in electron micrographs. It would be of interest now to explore in frogs bearing tumors without nuclear inclusions, whether treatment with carcinogenic or mutagenic agents would result in the appearance of the characteristic particles described here.

The tumor transmission experiments of Lucké, and those of Duryee as well, were apparently carried out without regard for the presence or absence of nuclear inclusions in the tumors used for preparing the inoculum. This may account for their low percentage of positive results (17 to 20 per cent). If infectivity depends upon the presence of the distinctive particles described here, one would expect to have little success with most of the tumors. On the other

hand, cell-free filtrates prepared from the occasional tumors that have abundant inclusions might prove to be far more effective. It is hoped that future efforts to induce tumors with cell-free filtrates will be designed to test this hypothesis.

SUMMARY

The common renal adenocarcinoma of the leopard frog was studied in thin sections with the electron microscope.

Approximately a third of the tumors examined were found to contain spheroidal bodies of uniform size and distinctive morphology that are believed to be virus particles. These consist of hollow spheres (90 to 100 m μ) having a thick capsule and a dense inner body (35 to 40 m μ) that is eccentrically placed within the central cavity (70 to 80 m μ). Virus particles of this kind occur principally in the cytoplasm but occasionally they are also found in the nucleus and in the extracellular spaces of the tumor.

The intranuclear inclusion bodies that are visible with the light microscope are largely comprised of hollow, spherical vesicles with thin limiting membranes. These are embedded in a finely granular matrix. A few of the thin walled vesicles contain a dense inner body like that of the cytoplasmic virus particles. This suggests that they may be immature virus particles. The inclusion bodies are believed to be formed in the course of virus multiplication but they usually contain very few mature virus particles.

Bundles of dense filaments and peculiar vacuolar inclusions also occur in the cytoplasm of the tumor cells. These seem to be related in some way to the presence of virus but their origin and significance remain obscure.

These findings are discussed in relation to previous work suggesting that the Lucké adenocarcinoma is caused by an organ-specific filtrable agent. It is concluded that the "virus particles" found in electron micrographs of the tumor cells *may be* the postulated tumor agent. On the other hand, the possibility remains that the particles described here are not those that are causally related to the tumors.

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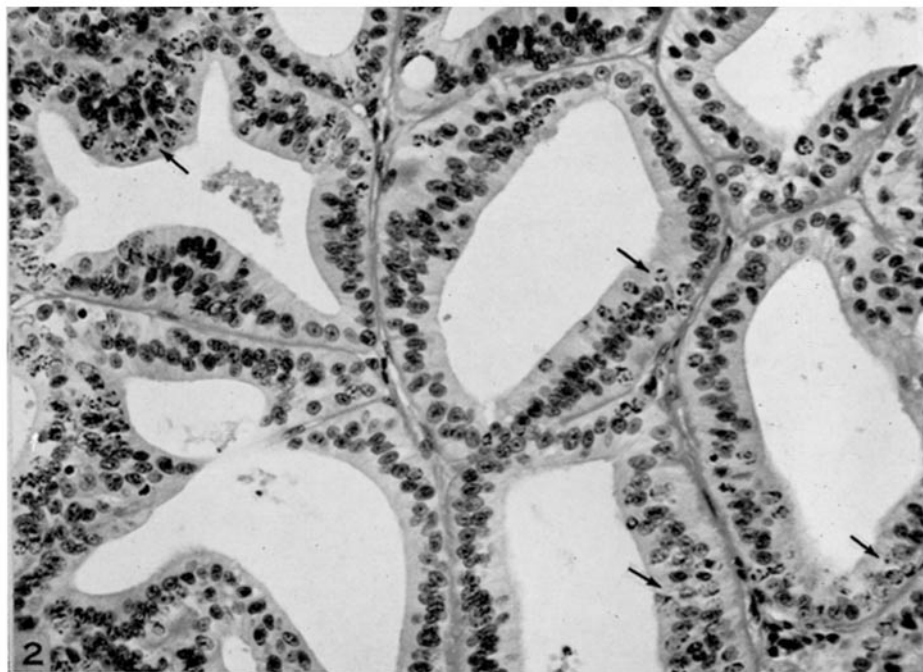
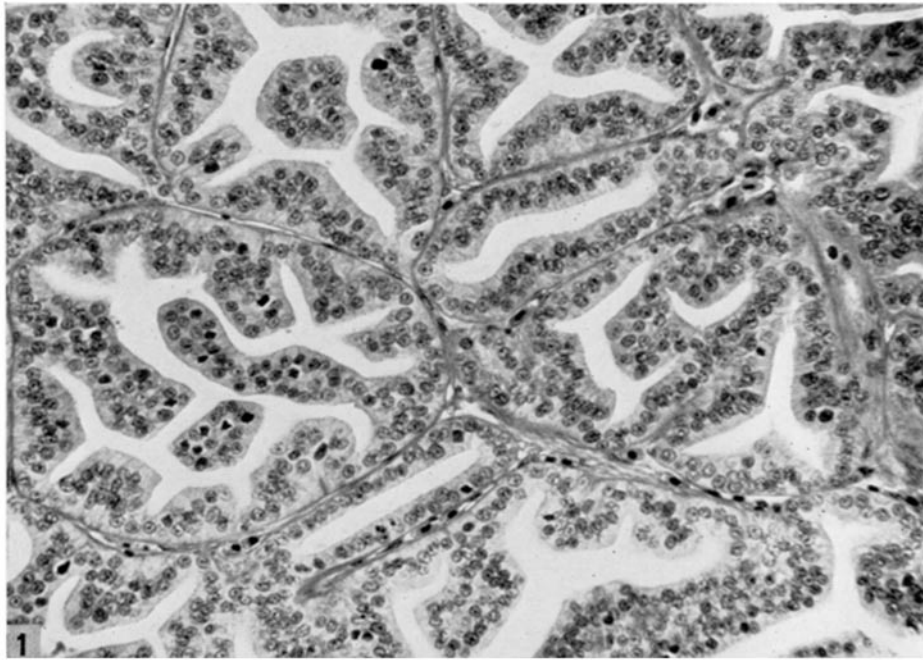
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EXPLANATION OF PLATES

PLATE 183

FIG. 1. Photomicrograph of typical renal adenocarcinoma consisting of columnar epithelium lining irregular alveolar spaces. No intranuclear inclusions are present. Approximately two-thirds of the tumors studied have been of this type. Feulgen reaction with light green counterstain. $\times 200$.

FIG. 2. Photomicrograph of a renal adenocarcinoma in which a considerable number of the cells show lightly stained inclusion bodies in the central portion of the nucleus and have their nucleolus and chromatin displaced to the periphery. The arrows indicate areas where such nuclei are to be found. About one-third of the tumors studied have shown nuclear changes of this kind. The electron micrographs on the following pages are from tumors of this type. Feulgen reaction counterstained with light green. $\times 200$.



(Fawcett: Electron microscopy of Lucké tumor)

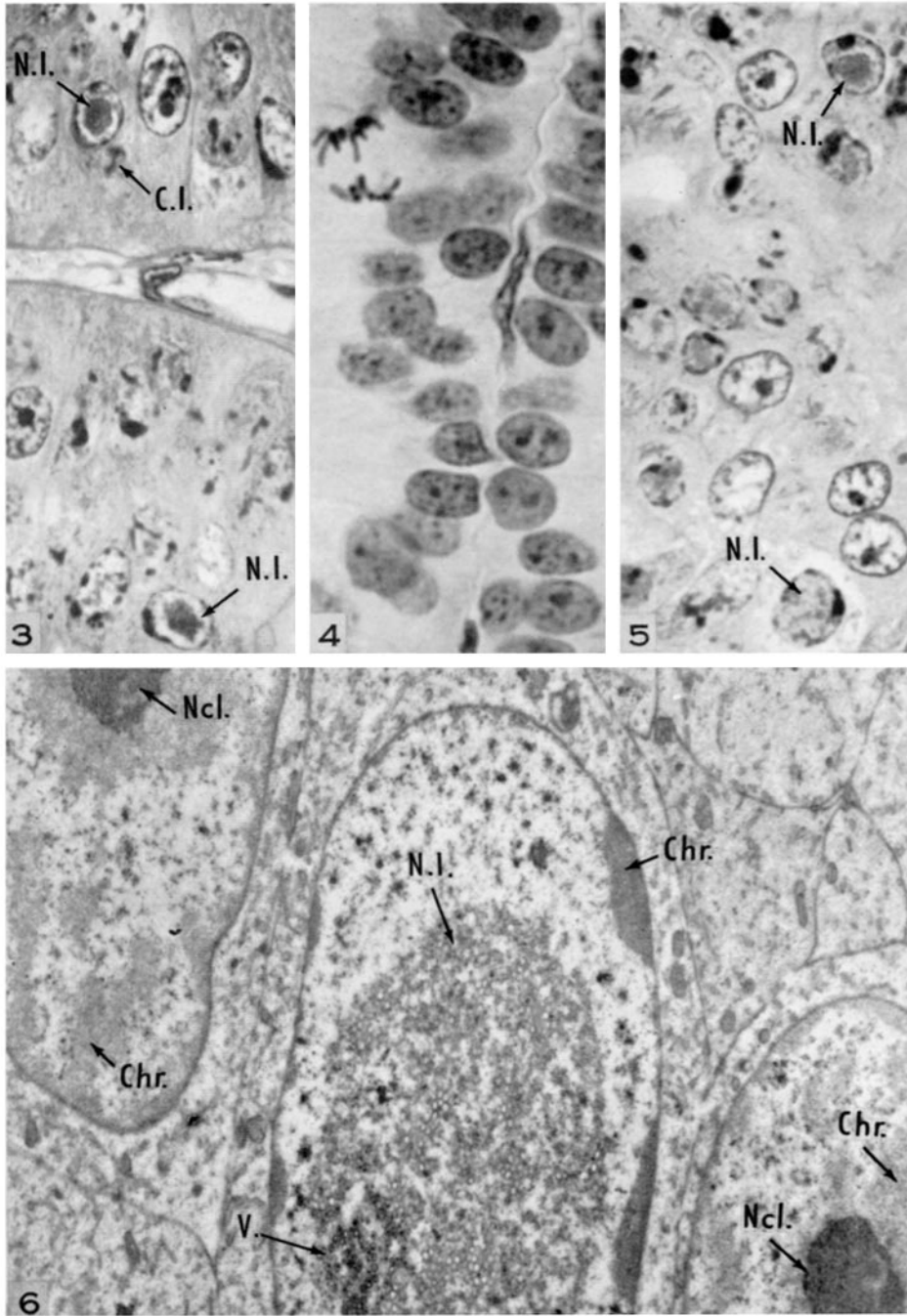
PLATE 184

FIG. 3. A photomicrograph of a renal tumor at higher magnification, including several cells with typical Type A nuclear inclusions (*N.I.*). Others show margination of chromatin but no single, sharply defined inclusion body. Irregularly shaped inclusions stained with hematoxylin are also found in the cytoplasm of some cells (*C.I.*). Chromium-alum-hematoxylin and phloxin. $\times 750$.

FIG. 4. Photomicrograph of a tumor showing no cytological evidence of virus infection. Nucleoli are centrally situated and chromatin clumps distributed throughout the nucleoplasm. Mitotic figures are generally more numerous in tumors of this kind than in those with abundant inclusions. Hematoxylin and eosin. $\times 975$.

FIG. 5. Same as Fig. 3 above. The nucleus at the upper right shows clearly a large nucleolus displaced against the nuclear membrane by an inclusion body (*N.I.*) in the center of the karyoplasm. $\times 750$.

FIG. 6. Electron micrograph at relatively low magnification showing portions of three tumor cell nuclei. Those at the upper left and lower right are normal in appearance with dense, compact nucleoli (*Ncl.*) and nebulous masses of chromatin of low density (*Chr.*). The nucleus in the center of the figure contains a large inclusion body (*N.I.*) and sharply defined, relatively dense masses of chromatin (*Chr.*) against the nuclear membrane. The inclusion consists, for the most part, of granular matrix and empty appearing vesicles of uniform size, but at its lower left is a cluster of mature virus particles (*V.*). $\times 6000$.

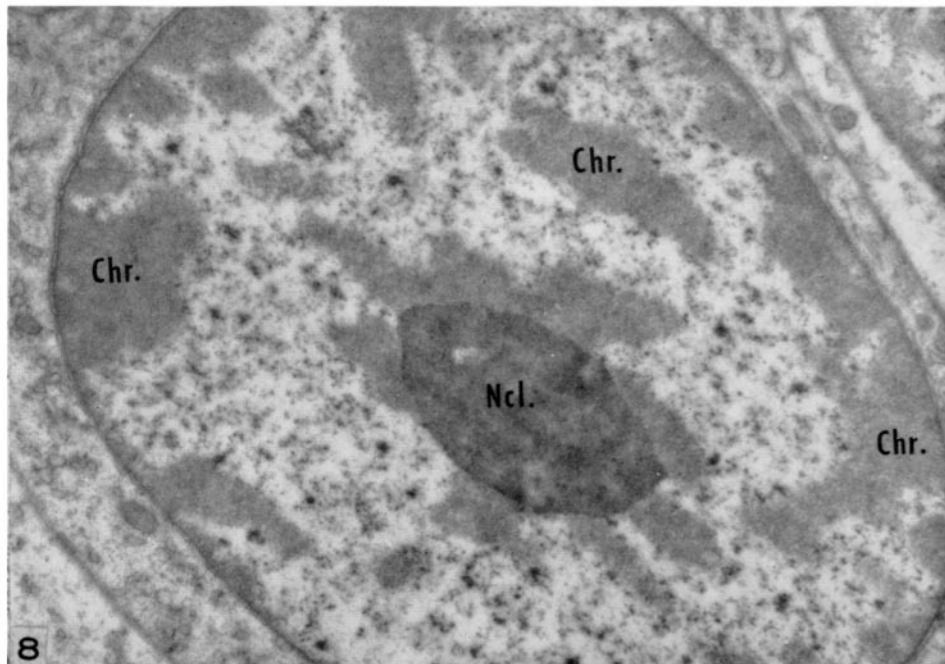
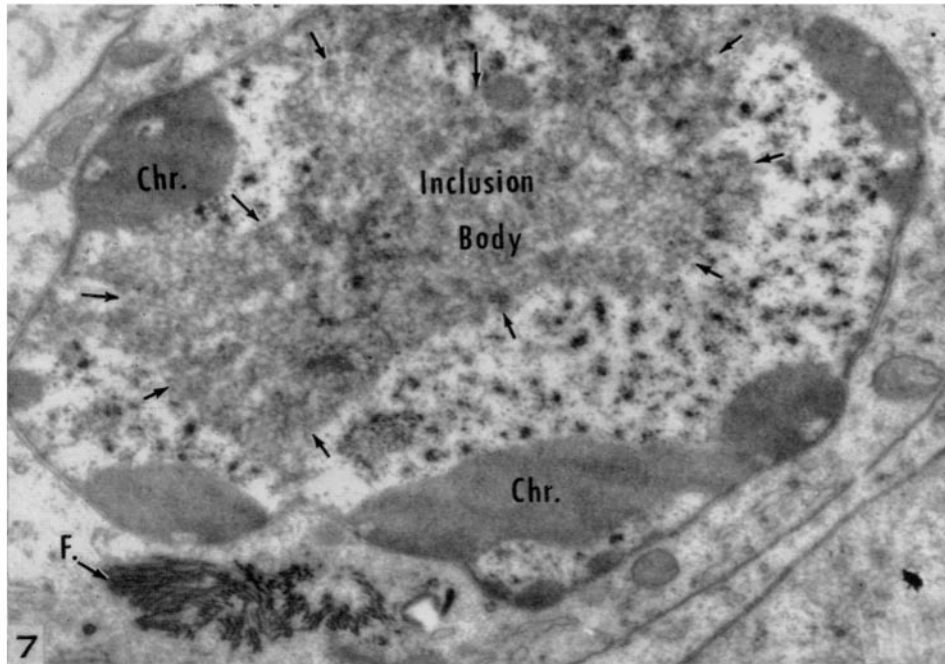


(Fawcett: Electron microscopy of Lucké tumor)

PLATE 185

FIG. 7. Electron micrograph of a tumor cell nucleus containing an inclusion body (outlined by arrows), composed of large numbers of hollow spheres of uniform size. The chromatin (*Chr.*) is in the form of large dense clumps adhering to the nuclear membrane. Numerous small aggregations of dense granules are seen scattered throughout the karyoplasm. A bundle of filaments (*F.*) is found in the juxtannuclear cytoplasm. In the lower right hand corner of the figure is part of an intranuclear inclusion of a neighboring cell showing the component vesicles arranged in straight rows. $\times 15,500$.

FIG. 8. An electron micrograph of a tumor cell nucleus that shows no obvious pathological changes. The conspicuous, irregularly shaped masses of fine granules that are distributed through the karyoplasm (*Chr.*) correspond to the clumps of chromatin visible in histological sections. The centrally placed nucleolus (*Ncl.*) is compact in its structure, sharply outlined, and is comprised of fine granules of about the same size as those of the chromatin but considerably denser. In addition, a few small clumps of granules of about the same density as those of the nucleolus are widely scattered in the karyoplasm. These latter are much more numerous in the abnormal nuclei (Fig. 7) and it may be that they are related to the presence of virus. $\times 14,000$.

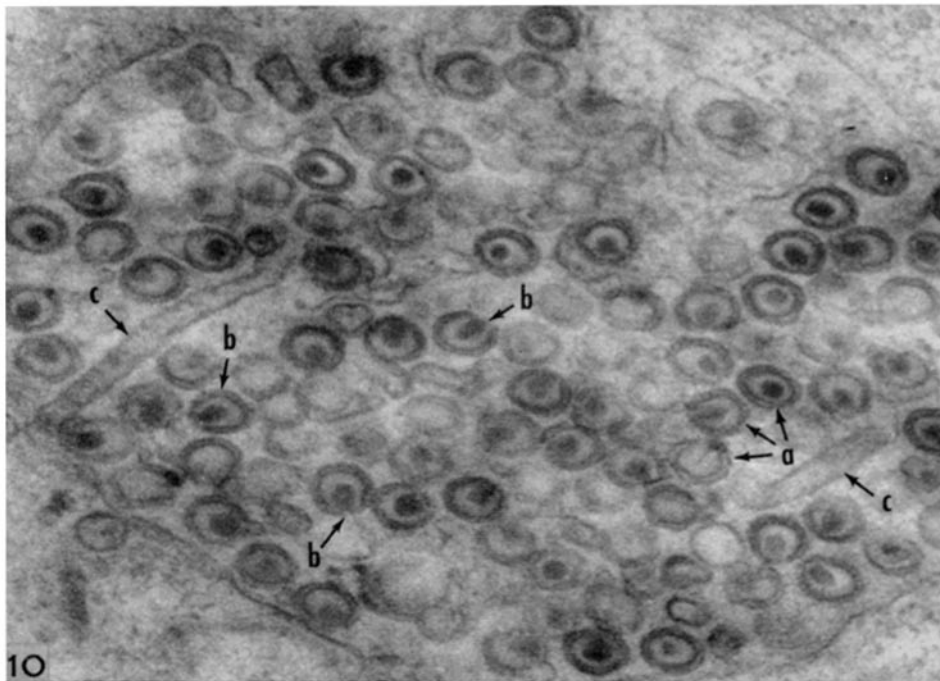
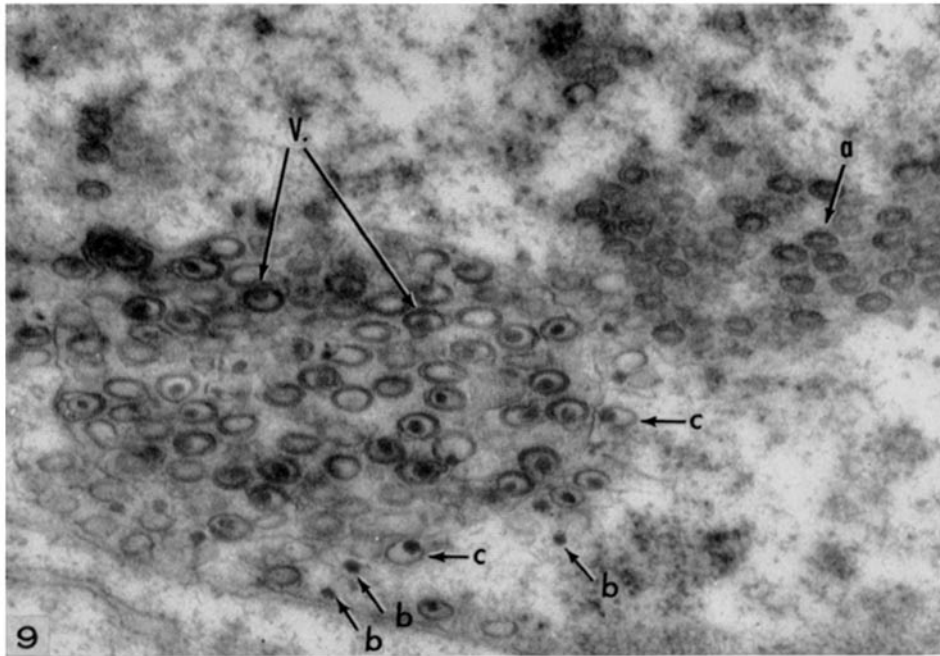


(Fawcett: Electron microscopy of Lucké tumor)

PLATE 186

FIG. 9. A peripheral portion of a tumor cell nucleus showing a collection of mature virus-like particles (*V.*) which seem to be enclosed by a delicate membrane. The majority of the particles have a thick dense capsule and a dense spherical body is visible in the interior of many of them. Outside of the membrane which surrounds this aggregation of particles, there are other particles of about the same size but with thin limiting membranes. A few of the dense spherical bodies are free in the karyoplasm (*b*) while others are partly within thin walled vesicles (*c*). At the right (*a*) is a cluster of smaller vesicular structures with double membranes and no dense inner bodies. $\times 50,000$.

FIG. 10. A large cluster of virus particles in the cytoplasm; the double nature of the capsule is clearly shown on the particles marked (*a*). The eccentric position of the dense inner body and its close relation to a thin area in the capsule are also visible in the particles marked (*b*). The tubular structures marked (*c*) are of rare occurrence and their relation to the virus particles is entirely unknown. $\times 66,000$.

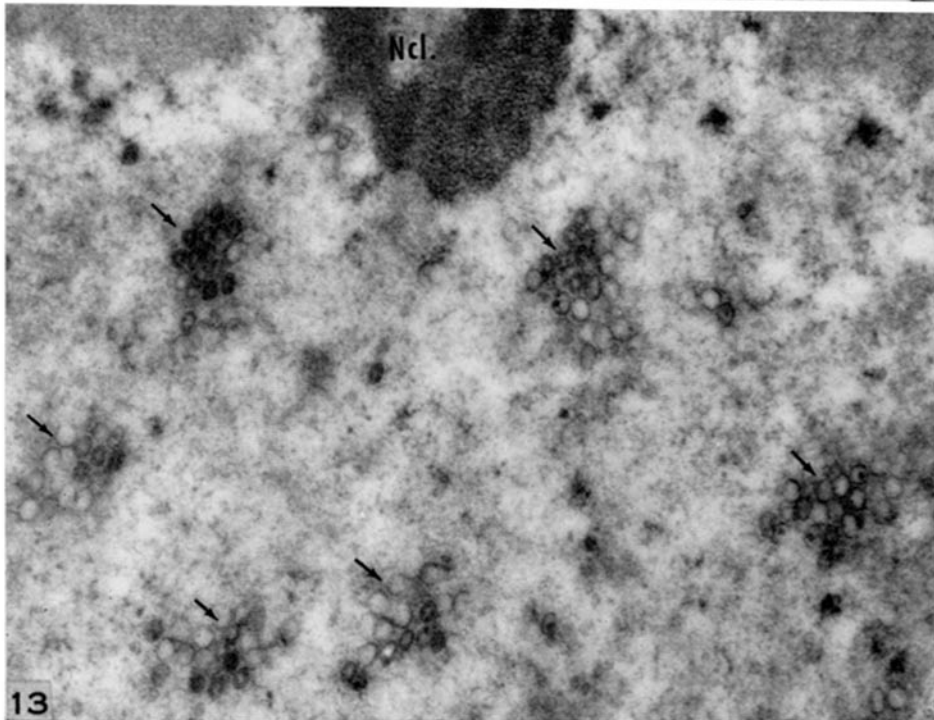
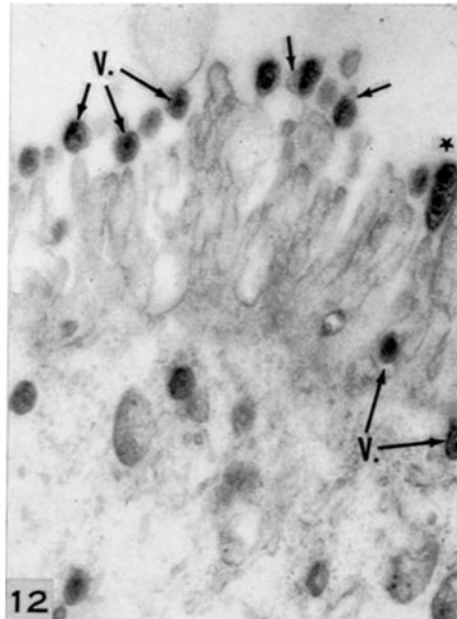
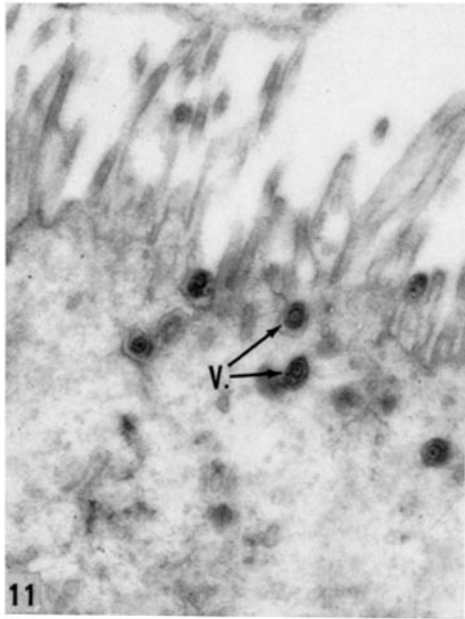


(Fawcett: Electron microscopy of Lucké tumor)

PLATE 187

FIGS. 11 and 12. Portions of the free surface of two cells lining spaces within the tumor. Virus particles (*V.*) occur in the cytoplasm and among the microvilli on the cell surface. The extracellular particles are surrounded by a thin outer membrane seen particularly well on particles indicated by arrows at the top of figure 12. At the right side of this figure (asterisk) the expanded tip of a microvillus containing two virus particles appears to be about to separate from the cell. Two or three of the intracellular particles in these figures happen to have a second thick capsule as do three of the particles depicted in Fig. 20, but this is unusual. $\times 20,000$

FIG. 13. An area of karyoplasm from a tumor cell showing early pathological changes believed to be associated with virus multiplication. The dark body at the top of the figure is the nucleolus (*Ncl.*). Many clusters of minute hollow spheres limited by either a single or a double membrane are present (see at arrows). These clusters may later become confluent to form a single large nuclear inclusion. $\times 35,000$.

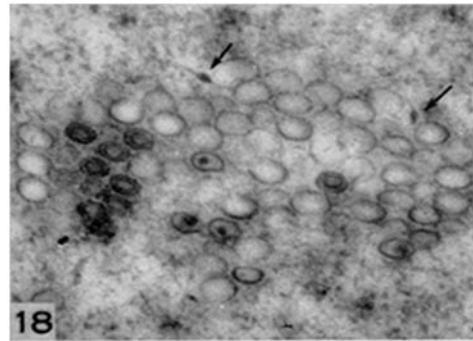
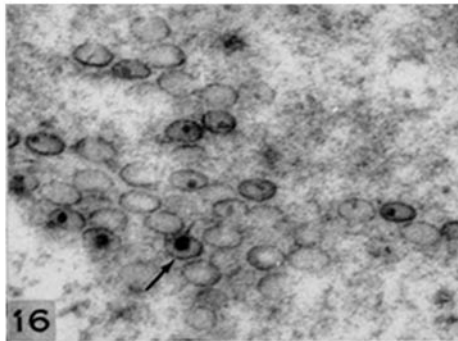
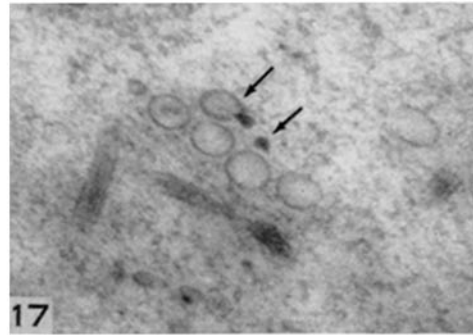
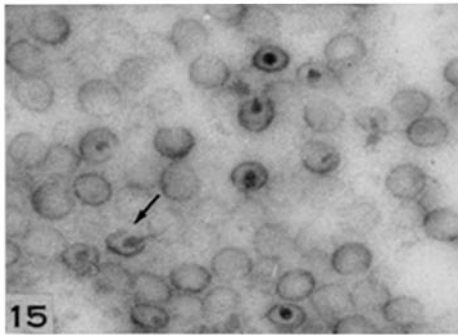
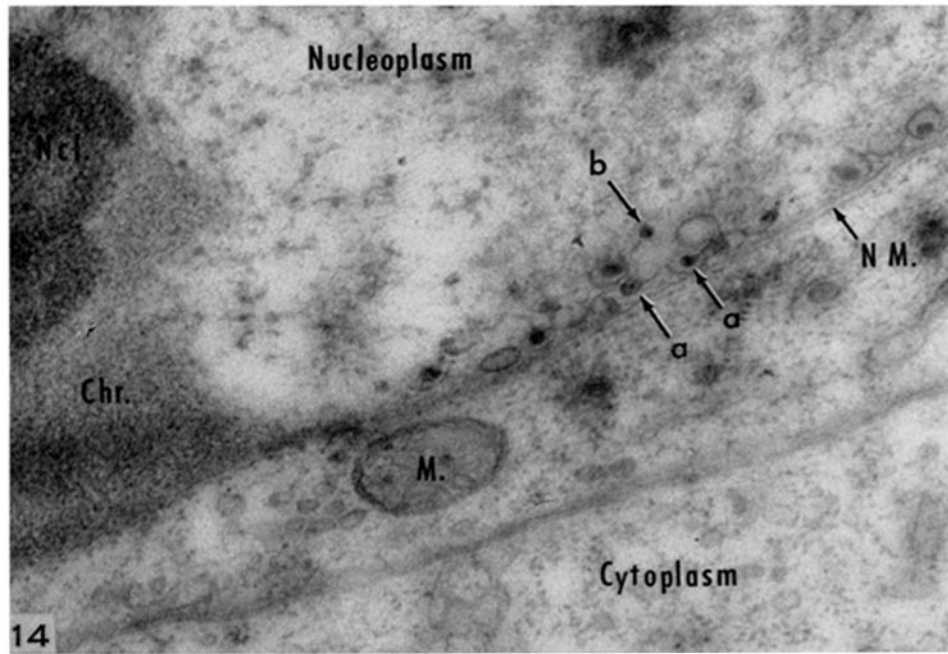


(Fawcett: Electron microscopy of Lucké tumor)

PLATE 188

FIG. 14. A portion of the periphery of a nucleus and an adjacent area of cytoplasm. Part of the nucleolus (*Ncl.*) and a clump of chromatin (*Chr.*) are visible at the left. The nuclear membrane (*NM.*) runs diagonally across the picture. Just within the nucleus are several thin walled vesicles, some appearing empty, others (*a*) containing a dense inner body with a slender, tail-like projection. These dense bodies also occur free in the karyoplasm without an enclosing membrane (*b*). $\times 53,000$.

FIGS. 15 to 18. Selected areas from inclusions within the karyoplasm of tumor cells showing various abnormal structures tentatively interpreted as components of developing virus particles. Observe that the membrane of the vesicles is often incomplete and the amorphous content is of very low density. In Fig. 15, at the arrow, a tadpole-shaped dense body projects into one of the vesicles through an opening in its membrane. Similar bodies are indicated by the arrows in the other figures. In Fig. 17 the slender tail-like process of one extends into the interior of one of the vesicles. In Fig. 18 two dense elliptical bodies with slender tail processes are seen lying free in the karyoplasm adjacent to a group of empty appearing vesicles. $\times 53,000, 50,000, 60,000, 46,000$.

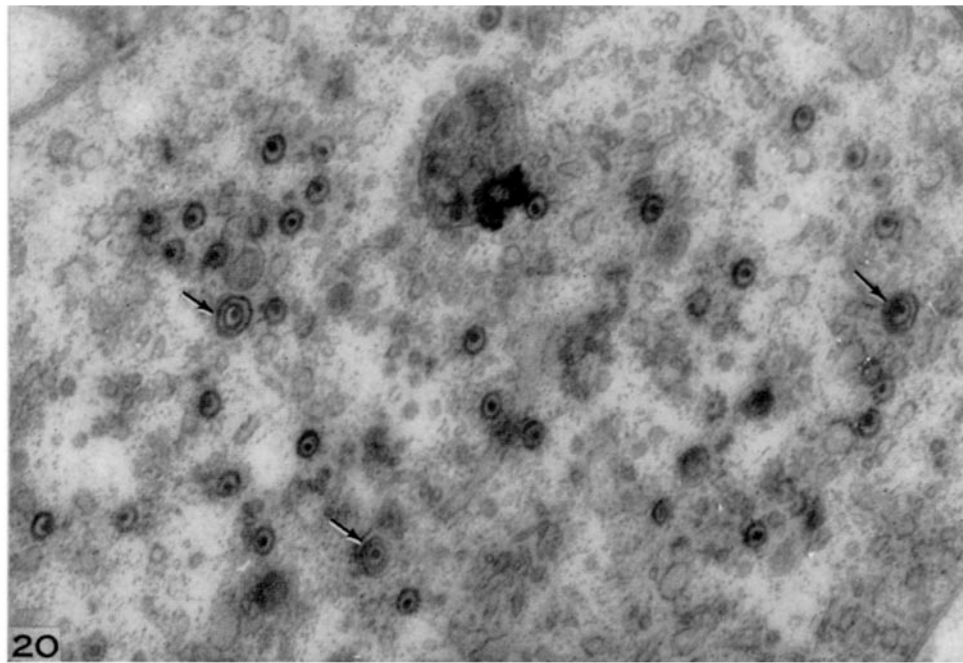
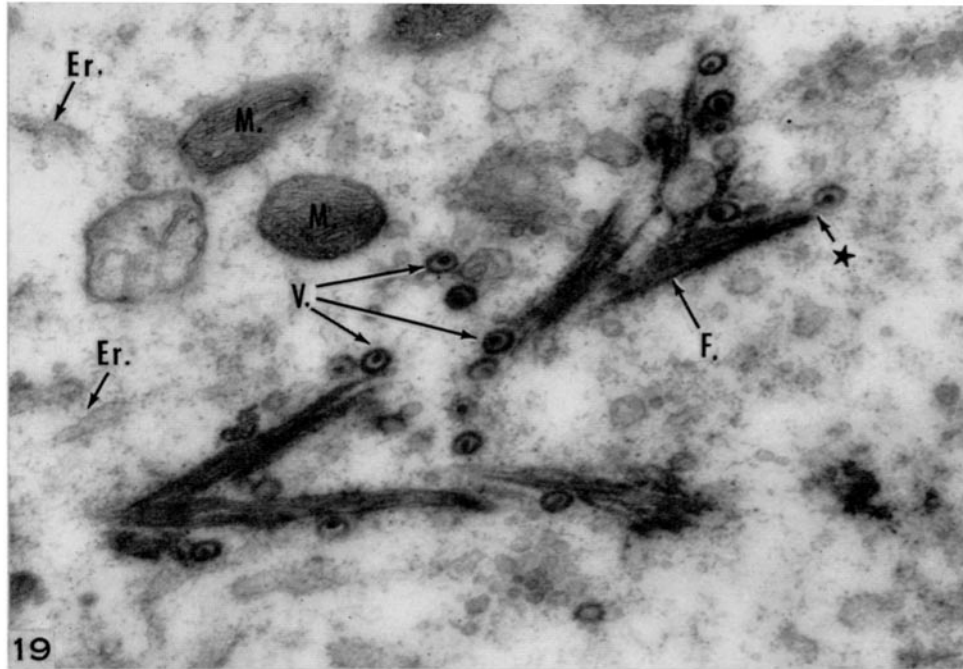


(Fawcett: Electron microscopy of Lucké tumor)

PLATE 189

FIG. 19. An electron micrograph of a small area of tumor cell cytoplasm containing mitochondria (*M.*), tubular strands of endoplasmic reticulum (*Er.*), virus particles (*V.*), and bundles of dense filaments (*F.*). The filaments are comprised of smaller fibrous subunits less than $10\text{ m}\mu$ in thickness. In the original micrograph at the point indicated by the asterisk, there appears to be continuity between two of these delicate fibers and the dense central body of one of the neighboring virus particles. $\times 35,000$.

FIG. 20. An area of cytoplasm containing a considerable number of virus particles. Three of these, indicated by arrows, are atypical in that they seem to have a second capsule outside of the usual one. $\times 30,000$.

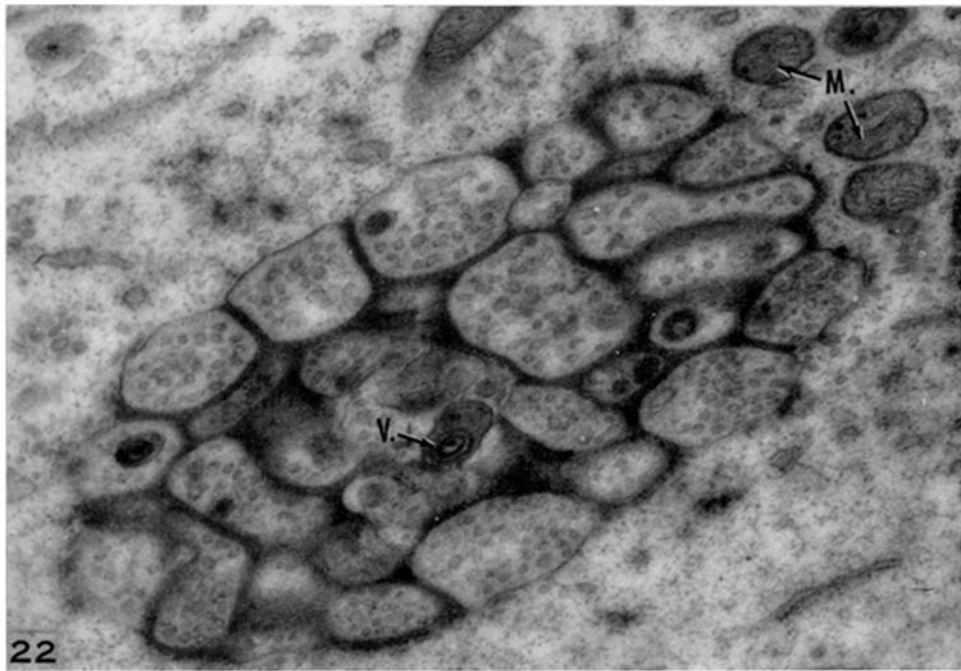
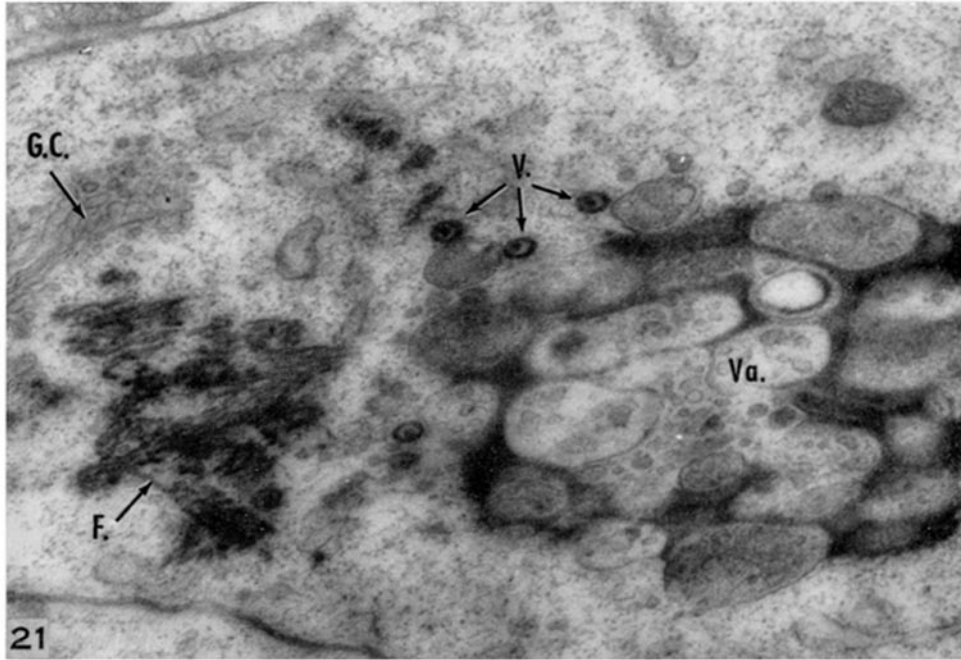


(Fawcett: Electron microscopy of Lucké tumor)

PLATE 190

FIG. 21. An area of cytoplasm in which the background is stippled with minute granules presumed to be the ribonucleoprotein responsible for the diffuse basophilia of the tumor cells. A portion of the Golgi complex (*G.C.*) is included in the field, also a cluster of dense filaments (*F.*) and a vacuolated inclusion (*Va.*). A few virus particles (*V.*) are also present. $\times 34,000$.

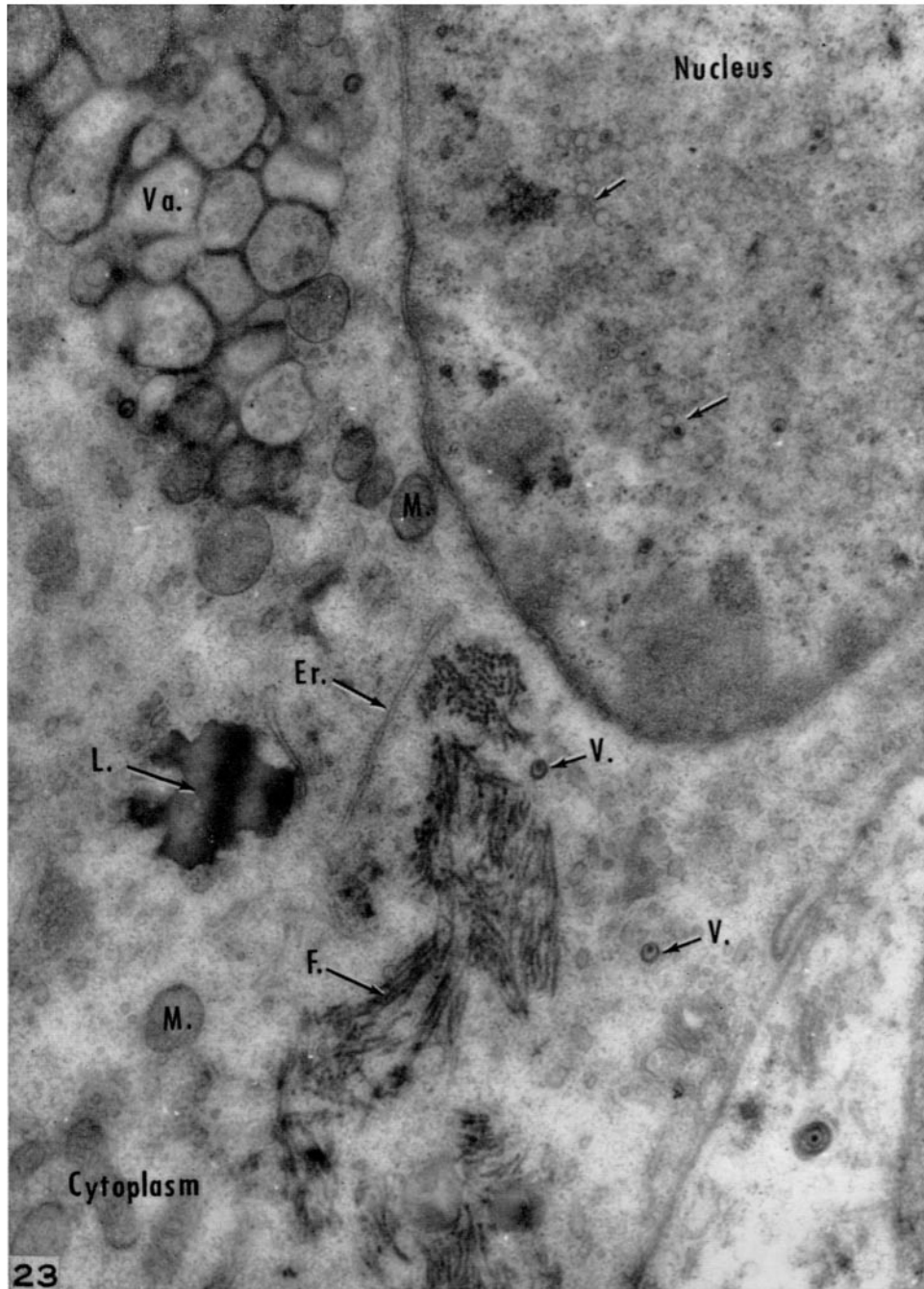
FIG. 22. An electron micrograph of a typical vacuolar cytoplasmic inclusion. The vacuoles of varying size and shape contain large numbers of small membrane-bounded vesicles. The presence of internal membrane-limited structures in the vacuoles suggests the possibility that they may arise from fusion of degenerating mitochondria or that they may be an abnormal product of the Golgi complex, but definite transitional forms establishing the origin of these inclusions from one or the other of these sources have not been observed. Typical mitochondria (*M.*) are seen at the upper right. A few isolated virus particles are occasionally found in the interior of the vacuolar inclusions (*V.*), but their occurrence there is believed to be accidental. $\times 34,000$.



(Fawcett: Electron microscopy of Lucké tumor)

PLATE 191

FIG. 23. An electron micrograph illustrating several of the normal and pathological components found in the tumor cells. The mitochondria (*M.*), the endoplasmic reticulum or ergastoplasm (*Er.*), and the lipide droplet (*L.*) are relatively normal in appearance. At the upper left, however, is an abnormal aggregation of vacuoles (*Va.*) that contain many small vesicles. The vacuoles are held together by a dense amorphous material deposited in the interstices between them. The bundles of dense filaments (*F.*) seen in the lower half of the figure are not found in normal cells. Scattered through the cytoplasm are virus particles (*V.*) which have a thick capsule delimiting a cavity that contains a dense inner body. In the nucleus at the upper right (at arrows) are many thin walled vesicles, in some of which an inner body is distinguishable. These vesicular structures in the nucleus are interpreted as developmental stages of the virus particles found in the cytoplasm. $\times 24,000$.



(Fawcett: Electron microscopy of Lucké tumor)