THE TOXIC EFFECTS OF TESTOSTERONE ON C3H MOUSE MAMMARY ADENOCARCINOMA IN ORGAN CULTURE

by

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INTRODUCTION

Testosterone is widely used to treat advanced mammary carcinoma in the human female. The mode of action of this hormone on neoplastic tissue, whether indirectly through some body process, or directly upon the neoplastic cells themselves, is essentially unknown. However, it should be possible to test for direct effects of hormones if the cancerous tissue is isolated from its complex host and maintained free of host influence.

A suitable biological system for this purpose, utilized in the present experiments, consists of mouse mammary carcinoma maintained *in vitro* in organ culture in a synthetic, chemically defined medium (76). Essentially pure hormones are added to the medium and the morphologic effects produced thereby are studied by light and electron microscopy. The historical review of the literature which follows deals with mammary tissue culture, organ culture techniques, and the effects of pure hormones on C3H mammary carcinoma *in vivo* and *in vitro*.

Early attempts to culture mouse mammary gland epithelium met with little success (65, 92, 93). As early as 1924, however, Maximow (69) was able to maintain rabbit mammary gland *in vitro* in complex (protein-enriched) media. This worker induced secretory and proliferative changes with various crude tissue extracts, some of which were derived from hormone producing

tissues.

Recent attempts have been much more successful. In 1949, while studying the development of mouse hair *in vitro* from explants of the ventral body walls of embryos, Hardy (43) observed the development of mammary gland rudiments to a stage morphologically similar to that found in seven day old mice. Comparable results were obtained by Balinsky (8). In 1957 Lasfargues (60) obtained excellent growth of normal adult mouse mammary gland epithelium in roller tube and plasma clot slide cultures. Lasfargues pre-treated the tissue with collagenase to digest the fatty stroma and enriched the medium with 50 per cent human placental serum. High concentrations of steroid hormones present in this serum were felt to be responsible for the excellent growth obtained.

A parallel development of organ culture techniques occurred. In 1920 Fell and Robinson (35) cultured isolated femora of fowl embryos in watch glasses on chick plasma-embryo extract clots. In 1936 Parker (82) unsuccessfully attempted to use a liquid medium for organ culture. Then, in 1954, Chen (20) successfully combined both ideas by employing a floating disc of lens paper to support the organ explant on the fluid culture medium.

In 1957 Elias (28) maintained mammary tissue of mice *in vitro*, using the Chen adaption to liquid medium of the watch

glass method of organ culture of Fell and Robinson, and employing the synthetic chemically-defined culture medium "199" of Morgan, Morton, and Parker (76). Elias was able to maintain the viability and secretory activity of explants of prelactating mammary glands obtained from adult mice of the C3H/Crgl strain. Maintenance for five days required enrichment of the medium with appropriate combinations of purified protein and steroid hormones, including Estrone, progesterone, cortisol, growth hormone, and mammotrophic hormone. The minimum essential maintainance hormones were cortisol and mammotrophic hormone. Hyperplastic alveolar nodules reacted similarly to normal prelactating tissue, although it was more responsive to the effective hormones. Without hormonal supplementation there was extensive degeneration of the alveoli and small terminal ducts after five days.

Subsequent to the work of Elias, several investigators employed similar techniques to define the effects of various pure protein and steroid hormones on organ cultures of mammary glands of embryonic, virgin and prelactating mice (29, 30, 62, 74, 88, 89, 90, 91, 103). Much of this work utilized the C3H/Crgl strain of mouse which normally bears a mammary tumor agent (MTA). In this strain almost 100 per cent of the breeding and virgin females develop spontaneous mammary adenocarcinoma as long as the agent is present. The use of mice in the study of the hormonal influences on the neoplastic transformation is facilitated by the presence of a

well recognized pre-neoplastic state --- the hyperplastic alveolar nodule. These hyperplastic areas have been extensively investigated (25).

Mouse mammary tumors frequently possess cytoplasmic inclusion bodies. According to Smoller et. al. (98), previous investigators concluded that the inclusion bodies were microscopically visible aggregates of the virus-like particles first demonstrated with the electron microscope by Porter and Thompson in 1948 (23) in an MTA bearing strain of mice. The virus-like particles are found not only in mammary tumors but also in hyperplastic alveolar nodules and infrequently in normal tissue (84). According to Pitelka, DeOme and Bern (85) an abundance of these particles are associated with hyperplasias of pre-neoplastic significance. The present state of knowledge of the hormonal influence in mouse mammary tumorigenesis, the characteristics of hyperplastic alveolar nodules, and the nature and significance of the mammary tumor agent have been recently reviewed (7, 13, 14, 15, 23, 25, 73). Previous work suggests that mouse mammary carcinomas are relatively independent of the hormonal milieu (68). Elias and Rivera (30) concluded that insulin has no effect on mammary tumor explants in organ culture as seen by light microscopy. Moretti and DeOme (74) have shown that the glucose uptake of mouse mammary carcinoma is independent of the presence of insulin, when the tissue is

maintained in medium 199 in organ culture. DeOme, et. al. (24) have observed no effect on radio-phosphorus uptake by mouse mammary carcinomas in vivo after administration of exogenous androgen, estrogen or cortisol.

Studies by Lasfargues, et. al. (61, 64) suggest that the mammary tumor agent (MTA) can be maintained in complex media in vitro and that it may actively multiple. The MTA appeared to maintain itself and actively multiple in organ cultures of developing embryonic mouse mammary glands and in roller tube cultures of partially digested adult mouse mammary tissue in which some adipose tissue was present. However, roller tube cultures of pure embryonic mouse epithelium failed to maintain the virus; maintainance without multiplication was achieved in cultures of pure epithelium from the adult mouse. Also, the virus-like particles can be found free in the medium in large numbers after 48 hours of primary explantation of tumor tissues and disappear about the 15th.-20th. day (63, 64). Lasfargues concludes that these facts support the contention that growth of the MTA requires the presence of some component of the primary tumor and/or epithelial-stromal interaction.

MTA may not be identical to the virus-like particles (VLP) seen in electron micrographs of mouse mammary carcinomas (73, 85). However, in vivo experiments demonstrate that

exogenous cortisone causes a significant increase in size and number of inclusion bodies by light microscopy and a corresponding increase in VLP by electron microscopy (98). There are as yet no reports to indicate whether this effect can be duplicated in vitro with cortisol or other hormones.

Several investigators have studied histologic specimens of human mammary carcinoma from patients' before and after steroid hormonal therapy. Adair and Herrmann (1, 48) have reported that hydropic degeneration of the cytoplasm and nuclear pyknosis were the main changes noted in human mammary carcinoma after testosterone therapy. Other observers have noted that cutaneous metastases from mammary carcinoma after testosterone therapy showed either no specific histologic changes (2, 106) or nondescript "degenerative changes" in the nucleus and cytoplasm of the cancer cells, which, however, seldom occurred to an unusual degree (2). According to Emerson et. al. (33), the morphologic changes occurring in mammary tumors regressing on steroid therapy are similar, regardless of whether androgens or estrogens are used. In two cases of mammary carcinoma, Haddow, et. al. (42) noted disappearance of mitoses and diminution of cellular staining affinity after estrogen therapy. In an addendum by Koller (59), after one month of stilbesterol therapy, the frequency of occurrence of potentially dividing Feulgen-positive chromocenters in tumor cell nuclei decreased from 12-27%

to 4.0-7.5%; cell division rate decreased from a maximum of 7.5% to 4.6%; and the frequency of degenerated cells increased from a maximum of 2.5% before therapy to 12% after one month of stilbesterol. Mitotic abnormalities consisting of thick short chromosomes which formed no equitorial plate at the metaphase, multi-polar spindles, and nucleolar vacuolation suggested that the primary effect of stilbesterol may be on the tumor cell nucleus. Hermann et. al (47) noted unusual nuclear irregularity and hyperchromatism, abundant mitoses, and unusually abundant, slightly granular cytoplasm in the one case of seventeen previously untreated mammary cancers which showed cytologic change following therapy with estradiol. Nothing unusual in the original tumor was noted. Emerson, et. al (31) and others (78) have observed that in the early stages of tumor regression after sex steroid hormone therapy, the degeneration of neoplastic cells was accompanied by fibrous proliferation and collections of plasma cells and lymphocytes near the necrotic foci. In 33 cases of primary operable breast carcinoma treated with estrogens or androgens for four weeks prior to radical mastectomy, Godwin and Escher (38) noted no pathognomonic histological changes although in some instances, cytoplasmic vacuolation was more prominent than might have been expected in untreated cases. Grossly, Nathanson (78) noted translucent, edematous softening of the tumor mass followed by shrinkage with an opaque, silky,

white, fibrotic appearance of the tumor mass after estrogen therapy. The corresponding microscopic picture consisted of edematous loosening and dissolution of the stroma surrounding the cancer cell clusters followed by separation of the cancer cells from the clusters so that they came to lie free in the tissue spaces and often showed nuclear and cytoplasmic "degenerative change". Lymph node metastases showed cystic degeneration of the tumor cells. Emerson, et. al. (32) made similar observations and concluded that hormonally induced regression involved both alteration of tumor stroma and destruction of tumor cells. The latter phase was characterized by tumor cells with swollen, pale, vacuolated cytoplasm and karyolytic or pyknotic nuclei. Fragmentation and disintegration of the cytoplasm was followed by similar degenerative changes in the nucleus.

Whether degenerating tumor cells disappeared by lysis or phagocytosis was not determined.

Data given regarding the specific hormones used for treatment, whether androgenic or estrogenic, was scanty. In a later report, Emerson (33) reaffirmed his previous findings and noted that in foci of primary breast cancers undergoing hormone induced regressions there is an apparent persistence of viable cancer cells particularly in lymphatics and ducts. Also, there was a slight tendency for neoplasms with more

abundant stroma to show more extensive hormonally induced repression. Most of the tumor successfully treated had scirrhous foci, and elastic tissue hyperplasia was marked in all but one of the tumors that responded to therapy.

By comparison, Huseby et. al. (54) noted that the effect of estrogen therapy on the normal breast tissue of patients with advanced mammary carcinoma is mainly stimulatory. In 34 of 36 treated postmenopausal patients an increase in cytoplasm and nuclear size of the epithelial cells occurred, with epithelial proliferation and elongation, and with extension and proliferation of small ducts. The formation of new lobules and proliferation of inter-lobular collagenous connective tissue was considerable. No correlation was found between the degree or type of epithelial cell proliferation and the response of the cancerous epithelium to the hormone.

Although both normal and neoplastic human mammary tissues have been grown in vitro by several different workers (11, 17, 19, 21, 36, 56, 80, 108), there are few experiments which explore the effects of testosterone on mammary cancer in vitro. Heuson and Legros (49) incubated mammary carcinoma tissue for 90 minutes in L-leucine-C^{14} in the absence and presence of varying concentrations of testosterone. They demonstrated a 21-36% inhibition of radioactive leucine uptake in the mammary

cancer tissue treated with concentrations of testosterone of 100 micrograms per 3 ml., compared with controls. Kellner and Turcic (57) cultured human mammary cancer cells for 72 hours in a medium containing 200 micrograms per ml. of testosterone. Variable results, sometimes a total explant destruction, and sometimes a simulatory effect similar to that obtained with low concentrations of estradiol, were obtained.

The effects of testosterone on the normal mammary gland of the rat were first reported by Selye, et. al. in 1936 (95). Crystalline synthetic testosterone benzoate injected in doses of 200 micrograms daily for 2 to 3 weeks caused a slight development of mammary tissue and marked milk secretion in immature male and female rats, both in the presence and in the absence of gonads. Recent studies (4, 5) reaffirmed this and showed that topically applied testosterone can stimulate lobulo-alveolar development in mature, gonadectomized male and female rats.

One of the first reports from which the effect of testosterone on rat mammary cancer could be implied was that of Heiman and Krehbiel in 1936 (46). They demonstrated that the percentage of successful homologous transplants of mammary fibroadenomas into male rats could be doubled by castration. Later, Heiman (44, 45) reported that injections of testosterone propionate not only significantly decreased the

percentage of takes of transplants of mammary fibroadenomata in rats, but it also markedly increased the frequency of transition of transplanted fibroadenomas to fibromas or sarcomas and inhibited the growth of the glandular components in transplanted fibroadenomata. Testosterone propionate was felt to inhibit the connective tissue elements of these tumors as well as the epithelial component whereas estrogen stimulated the epithelium and did not inhibit the connective tissue elements. Millar and Noble (70) also noted that testosterone had a suppressive effect on the growth of grafted rat tumors. Mohs (71) on the other hand, could demonstrate no effect of physiological levels of androgens on the transplantability and rate of growth of rat mammary fibroma and adenofibroma. Also, no effect on the percentage of takes of tumor transplants was noted either in castrates of either sex receiving esogenous testosterone or in castrated males receiving no exogenous testosterone (72).

Dunning (27) recently reported that testosterone treatment not only stimulated growth of transplanted R3230 rat mammary adenocarcinoma, but appeared to stimulate stromal growth while reducing the glands to a relatively benign appearing single layered structure. Tumors transplanted into males characteristically developed areas of metaplastic squamous epithelium with occasional squamous pearl formation. Shay, et. al. (96) noted that methyl-cholanthrene induced mammary tumors in male rats

or in testosterone treated female rats were predominantly of the spindle cell and collagenous type, in contrast to a predominant glandular tumor form in female rats or male rats given estradiol. Tumors developing in gonadectomized male and female rats in which male and female sex hormones were experimentally balanced tended to be of the mixed or fibroadenomatous form.

Huggins and co-workers (51, 52) have shown that while testosterone and many testosterone-derivatives can profoundly retard the growth of transplanted benign mammary fibroadenomata of the rat, certain steroids closely related to these androstane inhibitors accelerate tumor growth. The nature of the effect, whether stimulatory or inhibitory, appears to depend on the molecular structure of the steroid. The androstane compounds which caused either the restraint or promotion of tumor growth, had the common property of inducing proliferation of the normal mammary gland components.

Glenn and co-workers (37) have demonstrated a consistent growth inhibitory effect of testosterone propionate injections on hormone sensitive rat mammary fibro-adenomas transplanted into other female Sprague-Dawley rats. They found no correlation between the effectiveness of a testosterone-derivative (androstane compound) as a tumor growth inhibiting agent and its androgenic activity. While mammary fibroadenomata of

Sprague-Dawley rats are generally hormone-sensitive tumors, they may become insensitive to the normal growth inhibitory effect of testosterone and some testosterone-derivatives, yet still remain sensitive to other testosterone-derivatives. Although the testosterone-resistant tumors often grew faster than the testosterone-sensitive tumors, their growth was still markedly depressed by combined andrenalectomy and ovariectomy. Testosterone proprionate was shown to merely halt the progression of tumor growth. It did not bring about death of the tumor cells, regression of tumor growth, or "markedly discernible histological differences between tumors from control and treated animals". Withdrawal of testosterone administration following marked testosterone induced inhibition of tumor growth, resulted in the tumor resuming its original growth pattern (37).

One of the first reports on the effect of testosterone on mouse mammary tumors was that of Nathanson et. al. in 1939 (79). Grossly tumor free 4 1/2 month old C3H female mice were given twice weekly injections of testosterone proprionate. A marked decrease in the incidence of mammary tumors was noted, although tumors once present grew at the normal rate. Testosterone administration to mice with pre-existing tumors had no effect on tumor growth rate. They concluded that

testosterone proprionate given before development of the mouse mammary tumor will prevent its development. However, once a mammary tumor is formed, it is unaffected by testosterone. Mühlbock (77) noted no tumor regression either in C3H mice with the milk factor or in C3Hf mice with a low incidence of spontaneous mammary tumors without the milk factor, regardless of treatment by castration, ovariectomy, or testosterone pellet implantation. Similarly, Sugiura and Sugiura (102) noted no effect of testosterone proprionate in vivo in mice against eleven different transplantable mouse tumors including mammary adenocarcinomas. More recently, Podiltchak (86) treated one group of virgin C3HA mice with testosterone proprionate from age 2 to 2 1/2 months until age 9 months, another group similarly beginning at age 7 months until age 15 months, and a third group with combined castration and androgen plus corticosteroid treatment, beginning at age 5 1/2 to 6 months. In the first group, some inhibition of spontaneous tumor development was noted, with the tumors in treated mice developing at 13 to 21 months compared with 8 to 14 months in controls. In the second group some delay in tumor appearance in treated mice was observed. A marked delay in tumor development and a decreased incidence of tumor formation was noted in the castrated mice treated with testosterone proprionate plus corticosteroids. Glenn, et. al. (37) injected suspensions of

mouse mammary carcinoma into 10 to 12 week old C3H male mice and began treatment one day later with testosterone proprionate. No effect on tumor growth rate was noted. In similar experiments progesterone, leuteotrophin, growth hormone, estradiol, deoxycorticosterone, and pregnant mares' serum gonadotropin had no significant effect on tumor growth rates. On the other hand, adrenocorticotrophin (ACTH), prednisolone, and cortisol delayed tumor growth, but once treatment was withdrawn the tumor proceeded to develop normally.

The results obtained from cortisone treatment of mice with transplanted mammary tumors are varied. Some workers have noted no inhibitory effect for cortisone acetate (12,101,102) or ACTH (39,99,104). Other workers have observed that cortisone acetate (3,39), cortisol (99), hydrocortisone acetate (94,99), ACTH (99), and prednisolone (94) inhibited mouse mammary tumor growth. Although cortisone acetate treatment has been shown to induce a significant increase in the frequency of metastatic spread of the mouse mammary tumor (3,12), even the growth of these metastases is inhibited by cortisone, similar to the original tumor (12). While complete tumor destruction is not observed, cortisone has been shown to induce morphologic changes in mouse mammary tumors including

hemorrhage and necrosis with disorganization of tumor architecture, detachment of cells, and nuclear pyknosis (67). The body tissue wasting effect of hydrocortisone acetate, cortisol, and ACTH can be counteracted by simultaneous administration of prolactin and/or growth hormone without altering the growth inhibitory effect on the tumor (66, 99). Martinez and Bittner (68) demonstrated that bilateral adrenalectomy alone or combined with ovariectomy produced mammary tumor regression in only a small percentage of a group of mice of different strains. Similar results occurred in hypophysectomized mice. The tumors in the large majority of mice continued to grow progressively in spite of such treatment.

Glenn, et. al. (37) after considerable testing, concluded that steroids active against the testosterone-responsive fibroadenomata of the rat were not active against the C3H mouse mammary adenocarcinoma. Likewise, steroids effective against the C3H mouse mammary tumors were not effective against the rat mammary fibroadenomata. Only one steroid was effective against the C3H mouse mammary adenocarcinoma and the testosterone-resistent rat mammary fibroadenomata, namely 6 alpha-methyl-9 alpha-fluoro-17 acetoxy-21-dioxyprednisolone. Further, only those C_{21} steroids that were effective glycogenic

agents in adrenalectomized rats were effective anti-tumor agents in C3H mice. Glenn et. al. concluded that inhibition may therefore be merely a metabolic effect and that the mouse quantitatively and qualitatively differs from the rat as far as C_{21} steroid effects are concerned. The levels of steroid necessary to induce 90% to 100% depression of mammary tumor growth in mice were near the toxic level for those substances and often produced sick hosts.

Rivera* noted toxic effects of many steroid hormones on organ cultures of C3H/Crgl mouse mammary tumors maintained for five days in basal medium 199, with hormones added. Cortisol, corticosterone, and deoxycorticosterone were toxic at concentrations of 100 micrograms per ml. Aldosterone and cholesterol were partially toxic at concentrations of 200 micrograms per ml. Progesterone was toxic at a concentration of 75 micrograms per ml., partially toxic at 5 micrograms per ml., and non-toxic at 1 microgram per ml. The acinar structure of the tumor was noted to be better preserved in adrenal steroid enriched media than

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* Personal communication, 1963.

ovarian steroid enriched media. Tumor explants cultured in steroid free 199 consistently showed central necrosis and a peripheral rim of viable cells; even very small explants showed a central necrotic portion. In the degenerating tissue treated with toxic doses of steroid hormones, not all cells were in the same stages of degeneration at the same time. The effects of testosterone and testosterone-derivatives on the growth of tumors in rats and mice has been summarized by Drill (26).

As mentioned earlier, testosterone is used in the treatment of mammary carcinoma in the human. Experimentally, in mice and rats it has been found to alter the course of tumor growth. However, it has not been well demonstrated that testosterone has any direct effect on mammary carcinoma cells. The main purpose of this experiment, then, is to attempt to demonstrate the direct effect of testosterone on mouse mammary carcinoma cells in vitro in a synthetic media, as manifested by cytomorphologic changes.

The specific aims of the experimental work are:

1. to determine whether or not insulin and/or cortisol help maintain the cytomorphologic characteristics of mouse mammary adenocarcinoma in organ culture.

2. determine whether or not either testosterone or cortisol has a toxic effect on mouse mammary carcinoma in organ culture in a chemically defined synthetic medium.

3. to determine whether or not the toxic effects of testosterone or other hormones, if present, can be modified by the addition of other hormones.

4. to determine, whether or not the toxic effects of testosterone, or other hormones if present, are associated with characteristic alterations in the fine structure of the tumor cells by electron microscopy.

5. to determine what effects, if any, the above procedures have on the characteristic virus-like particles previously described in electron micrographs of these tumors.

Materials and Methods:

Retired female breeder mice of the mammary tumor bearing, mammary tumor virus-infected C3H/Crgl or C3H/JAX strains were used. Four to six animals were kept in each cage. The diet consisted of Purina mouse breeder chow and water, ad lib. Mice with tumors measuring 0.5-0.8 cm. were selected for culture, anaesthetized with ether or intra-peritoneal sodium pentobarbital (83), washed with 70% or 95% ethanol, and pinned out on a dissecting board. A midline, ventral skin incision extending from the tail to the head was made using sterile instruments, and the skin was carefully retracted to expose the tumor. A small (0.5 cm.) fragment of tumor was removed using a new set of sterile instruments and transferred to a deep well Maximow slide containing medium 199 with added antibiotics. The slide was placed in a Petri dish to protect its contents from dust, etc. The most desirable tumors for culture were 0.5-0.8 cm. in diameter or smaller, firm, pink-white, and free of abscesses or hemorrhages. Explants from such tumors were generally composed of uniform glandular parenchyma without scar tissue.

Using a binocular dissecting microscope, fine forceps, and cataract sissors, explants of mammary carcinoma measuring 1/2 to 1 mm. in diameter were cut from the fragment of tumor. Dissection was carried out on the ground glass surface of the Maximow slides with the tissue emersed in 199 (with antibiotics) to prevent drying.

Explants were kept on the ground glass slide surface in a drop of medium until a sufficient number had been cut to prepare all of the culture plates.

Each culture plate consisted of a 9 cm. Petri dish with 3 pieces of Whatman #2 filter paper on the bottom dish (fig. 1). A pyrex watch glass, measuring 6 cm. in diameter was placed on the filter paper. One cc. of culture medium containing the desired concentrations and combinations of hormones, cholesterol, ethanol and antibiotics was placed in the watch glass. A circle of silicone treated, special lens paper, measuring 2.5 cm. in diameter, was placed on top of the medium. The explants were carefully positioned on the lens paper in order to achieve uniform spacing. Each plate had 4 to 6 explants (fig. 1). The surface of the lens paper float was gently pressed with the forceps to aid penetration of the underlying medium through the pores of the lens paper. This was done in order to obtain a 1 to 2 mm. ring of medium around the explant, with the meniscus of the medium just reaching the upper surface of the explant (fig. 1). These details were checked with the binocular microscope. Transfer of the explants was done most easily by picking them up in a drop of fluid in the tips of the fine forceps, then lightly touching them to the top of the filter paper float. The forcep tips were kept far enough apart so as not to crush the explant.

Plastic spacers, 1 mm. thick, were used to separate the halves

of the Petri dish to facilitate gaseous change (fig. 1). The prepared plates were then placed in a polyethylene tray with a transparent air-tight cover. Each tray accommodated 6 to 12 plates. The trays were then placed in a water-jacketed copper incubator maintained at 35° to 37° C. The tray was continuously perfused with a mixture of 95% oxygen and 5% carbon dioxide, at a rate sufficient to maintain the pH of the culture medium at 7.2 to 7.6 as judged by color comparison of its phenol red indicator with known standards. Humidification in the culture vessel was maintained by bubbling the gaseous mixture through sterile distilled water prior to passing it through the culture chamber and by saturating the Whatman filter paper on the bottom of the Petri dish with approximately 4 ml. of sterile distilled water. Some preliminary cultures were incubated for periods up to 12 days and the medium was changed at 3 day intervals. The medium change was accomplished as follows: The filter paper float was first gently teased to the side of the watch glass with the needle tip of a disposable plastic syringe. The medium was removed from the watch glass with the syringe. Fresh medium was then added with clean, sterile syringes and the float was teased back into position. All of the organ cultures reported in this paper were for periods of three days or less, so no medium change was necessary.

For light microscopy, the lens paper floats with tissue still

attached were transferred with the aid of forceps to a clean vial to which Bouin's fixative solution was added. The tissue was fixed for 8 to 12 hours and dehydrated according to the following schedule: 70% ethanol for 8 to 12 hours, 95% ethanol for 1/2 to 1 hour, 100% ethanol for 1/2 to 1 hour, 100% toluol for 1/2 to 1 hour and molten embedding wax for 2 to 4 hours. The wax used consisted of nine parts of histowax (melting point of 54° to 56° C) and one part of bayberry wax. Eosin was added to the 70% alcohol used in the initial dehydration step in order to lightly stain the explants and render them more visible; this procedure minimized tissue loss during the dehydrating and embedding process. For convenience, the tissues may be stored in 70% alcohol for several days without ill effects. Tissues were embedded in wax in disposable plastic molds, and serial sections were cut at 7 microns. Entire explants were serially sectioned and mounted.

For electron microscopy the lens paper floats with attached tissue explants were first removed from the medium and placed on a glass plate. The explants were then teased from the filter paper with a spatula and placed in cool 1% osmium tetroxide for 2 hours. Dehydration was accomplished in 70% ethanol for 20 minutes, then 95% ethanol. The tissues were stored in 95% ethanol for one to seven days, embedded in Epon, sectioned at 50 to 100 millimicrons, and viewed in an RCA-EMU 3G electron microscope.

The following procedures were used for washing and sterilizing glassware. All glassware was rinsed in tap water immediately after use. Pipettes and watch glasses were treated with alcoholic potassium hydroxide solution for one hour. The alcoholic potassium hydroxide was prepared by dissolving 100 gms. of reagent grade potassium hydroxide in 100 ml. of distilled deionized tap water, then diluting 1:10 with 95% ethanol. The glassware was rinsed again in tap water and boiled for two hours in a stainless steel bucket containing tap water and 7 to 10 gms. of sodium carbonate. The bucket and its contents were allowed to cool overnight, the surface scum was floated off, and the glassware was rinsed several times with tapwater added to the bucket. Each individual Petri dish was rinsed 15 to 20 times on both surfaces under rapidly flowing tap water, and then placed in a drying rack. Clean rubber gloves w ere used for handling and rinsing glassware. The pipettes were rinsed in a pipette washer for one to two hours and then dried in an oven. The watch glasses were rinsed under rapidly flowing tap water with gloved hands for about 1/2 minute, followed by 12-15 rinses with distilled, deionized tap water. The watch glasses were then soaked for about 1 hour in two or three changes of distilled, deionized tap water, and placed on a rack to dry. The deep well Maximow slides were washed and rinsed in the same manner as the watch glasses. After drying, the culture and

dissecting plates were assembled with gloved hands and placed in stainless steel canisters. Two dissecting plates (each with two Maximow slides) and eight or ten culture plates occupied each canister. The 10 ml. and 1 ml. pipettes were placed in separate steel canisters. All glassware was then sterilized by dry heat in closed canisters at 160° C to 180° C for four hours.

Circles (floats) of lens paper (G.T. Garr, Ltd., London) measuring about 22mm. in diameter were made by means of a cork punch. The floats were treated according to the following procedure: (1) The floats were separated and spread out on the bottoms of Petri dishes which had been washed by the procedure previously described. About 20 floats were placed in each dish to avoid excessive overlapping and crowding. (2) Floats were washed under a hood in three changes of anhydrous ether (reagent grade) changed at one-half hour intervals. A hood was not necessary if the dishes were tightly covered with aluminum foil, and the room was well ventilated. Containers were agitated briefly at 5 to 10 minute intervals during all washing periods. Wash solutions were changed by careful decanting. (3) Floats were washed in three changes of 95% ethanol, changed at one-half hour intervals. (4) Floats were washed in five changes of distilled deionized tap water, changed at fifteen minute intervals. (5) The floats were allowed to

soak overnight in distilled, deionized tap water. (6) Floats were then rinsed in a 1 to 1000 dilution of a water-soluble silicone ("Siliclad", Clay-Adams, Inc., New York) for about 30 seconds, or until completely permeated by the solution. (7) Steps 4 and 5 were then repeated. (8) The floats were then dried in the incubator at 37° C overnight, stored in a clean, covered Petri dish, and sterilized immediately prior to use. Sterilization was accomplished by dry heat for two hours at 160° C.

The hormones used were amorphous insulin (Eli Lilly & Co., lot number W-1282, activity = 21 units per mg.), estradiol-17 beta (Schering Corp., lot number 1-RMA-97, MS-51975), progesterone (Schering Corp., Lot 1-RMA-74), cortisol (Merck, Sharp & Dohme), and crystalline testosterone (Schering Corp., lot number MH-6-ER-1-X). The steroids were in the unesterified form. Ash free, chemically pure cholesterol was used. The appropriate amounts of cholesterol or hormone, usually 1 or 2 mg., were weighed in acetone-washed, aluminum foil, disposable pans on a Cahn Electrobalance. All measurements were accurate to within 0.04 mg. After weighing, the substances were carefully transferred with acetone-washed forceps to sterile disposable polyethylene test tubes. Each tube was covered with an air tight sealing cap and stored at room temperature until used. For individual experiments, stock solutions of the desired substances were made by bringing them into

solution with a few drops of the appropriate agent, and then diluting to the desired concentrations with culture medium containing antibiotics. For steroid hormones and cholesterol, approximately 0.03 to 0.05 ml. of 100% ethanol was needed to dissolve 1.0 mg. of hormone. For amorphous insulin, approximately 0.02 to 0.03 ml. of freshly prepared 0.0001 normal hydrochloric acid was sufficient to dissolve 1.0 mg. of hormone. The dilute hydrochloric acid solution was freshly prepared each time using a clean, sterile 100 ml. volumetric flask and 1 ml. pipette. One ml. of a 1.00 N stock solution of hydrochloric acid, stored in a plastic bottle, was serially diluted to obtain the desired concentrations. Only sterile distilled deionized water was used.

The tissue culture medium employed was synthetic "199" (76) with phenol red and bicarbonate buffer added (obtained from Microbiological Associates or Hyland Laboratories). The medium was received in 100 ml. bottles. A fresh bottle was used for each experiment.

Vials containing one million units of potassium penicillin-G and 1 gm. of streptomycin sulfate were obtained from the hospital pharmacy. The antibiotics were diluted with medium 199 or with saline or distilled water to make a stock solution containing 10,000 units of potassium penicillin-G and/or 0.01 gms. of streptomycin sulfate per ml. A 1 ml. aliquot of this solution was then added to

a fresh bottle of medium 199 containing 99 or 100 cc. of fluid. Thus, a final antibiotic concentration of about 100 units of penicillin and/or micrograms of streptomycin per ml. of medium 199 was obtained.

All serial dilutions and hormone mixtures were prepared in a tissue culture hood, using washed sterile pipettes and sterile disposable polyethylene test tubes. Sterile, disposable syringes and needles were employed to transfer the culture medium to the watch glasses. The tissue culture hood and surrounding counter areas, shelves and floor were washed with 70% ethanol immediately before an experiment was done. The hood itself contained an ultraviolet light which was left on when the hood was not in use.

The order in which the parts of the experiment were usually carried out was as follows: (1) Plan the experiment, including the number of plates to be used and the hormones needed. (2) Prepare and sterilize the necessary plates and filter paper floats. (3) Weigh the needed hormones. (4) Wash the tissue culture hood, counters, and floor with 70% alcohol. (5) Prepare the medium and the hormone mixtures. (6) Prepare the tissue culture plates. a. Add 4 cc. of sterile distilled water to soak the Whatman filter paper in each culture plate. b. Add 1 ml. of the culture medium to the watch glass. c. Place the siliconized filter paper float on the medium in the watch glass. d. Place the alcohol sterilized, air-dried, plastic spacer over the rim of the Petri dish. e. Place the

plates in the polyethylene tray and equilibrate in the incubator with respect to the gaseous mixture, humidity, temperature, and pH. f. Select and anesthetize the mouse. Prepare the necessary explants. h. Load the culture plates and return them to the incubator.

The following is a brief summary of the material and methods. The Chen modification of the watch glass technique of organ culture of Fell and Robinson was used. Sterile explants of mouse mammary carcinoma measuring 1/2 to 1 mm. in diameter were obtained from retired breeders of the C3H strain. The explants were maintained in organ cultures for periods up to three days using chemically defined medium 199, both with and without added hormones. At the end of the culture period, the explants were harvested and prepared for viewing. For light microscopy, tissues were fixed in Bouin's solution, stained with hematoxylin and eosin, and serial sectioned in their entirety. For electron microscopy, tissues were fixed in buffered 1% osmium tetroxide and embedded in Epon. By light microscopy morphologic changes were evaluated so that degree of survival of the explant could be estimated. Criteria for judging explant survival were (1) maintainance of the architecture of the tumor,and (2) the relative number of karyolytic, pyknotic, and karyorrhexic nuclei. A rating scale from 0 to 6 was used, with 0 representing complete necrosis and 6 representing complete survival. Allowances were made on the basis of experience in observing large numbers of explants

for the not uncommon finding of central necrosis and/or peripheral necrosis. Peripheral necrosis is probably due to submersion in the fluid medium and/or trauma in handling, while central necrosis may be due to relatively poor diffusion of nutrient culture medium and oxygen into the central portion of the explant mass.

Results: Light Microscopy The morphological effects of the various hormonal combinations and concentrations were evaluated by comparing the histology of the explants with the original, untreated mouse mammary adenocarcinomas. The histological patterns of the original 10 tumors used for the present experiment varied considerably. Most tumors formed acini; some formed cysts, solid nests, sheets, trabeculae, papillary formations, or various combinations of these (fig. 2-9).

Relatively small tumors, measuring 0.5-0.8 cm., were selected for organ culture when available. Tumors of this size range were composed predominantly of acini or solid nests with very little stroma. Cross sections of small acini revealed about 8-12 cells of relatively uniform size arranged about a central lumen (fig. 2, 6). Cytoplasm was eosinophilic and finely granular. Occasional empty cytoplasmic vacuoles were present; these presumably represented lipid droplets removed by fat solvents during the preparation of the tissues. Nuclei were usually round or oval and varied slightly in size. Chromatin was deeply basophilic and finely granular. Nucleoli were frequently inconspicuous. Mitotic figures were numerous and occasionally abnormal. Pyknotic, karyolytic, and karyorrhexic nuclei were rare. Acinar lumens sometimes contained small amounts of eosinophilic material.

Older neoplasms (over 1 cm. in diameter) were less desirable for organ culture because they showed more variable histological patterns,

degenerative changes, and uneven stromal fibrosis (fig. 5, 7-9). Large zones of necrosis and hemorrhage were sometimes present. Although these zones were avoided in selecting tissue for explants, a greater number of karyolytic, pyknotic, and karyorrhexic nuclei were observed than in the small tumors. Many of the older tumors were composed largely of solid nests or sheets of epithelial cells. Some had extensive zones of stromal fibrosis. In general the quantity of fibrous tissue in the stroma appeared to increase with the size of the tumor. Epithelial "pearls", consisting of concentric whorls of flattened cells with eosinophilic cytoplasm, were occasionally observed. The "pearls" were usually found in the central portions of solid cell nests or acini (fig. 9).

Figure 10 depicts normal mouse mammary gland for comparison with the sections of adenocarcinomas shown in figures 2 to 9.

Control tissue explants were maintained for 1 to 3 day periods in medium 199 alone (fig. 11), in medium 199 plus 0.8% or less ethanol (fig. 12), or medium 199 plus 0.3% ethanol plus 50 micrograms per ml. of cholesterol (fig. 13). These control preparations were histologically indistinguishable from each other and differed from the original tumor only in that a few scattered karyolytic and pyknotic nuclei were present. Tissue patterns (acinar, solid nests, trabecular, etc.) were well maintained throughout the culture period.

In addition, the control cultures and cultures maintained in the lower concentrations of testosterone (0.1 - 0.1 micrograms per ml.)

sometimes showed very subtle morphological changes which were judged to be a non-specific effect, perhaps due to the culture procedure (fig. 11-15). Nuclei were sometimes slightly swollen with an increased coarseness of their chromatin pattern. Both nuclei and cytoplasm were sometimes paler staining than in the original tumors. These changes were readily distinguishable from the prominent karyolysis observed in the cultures maintained in testosterone (e.g., fig. 16), and tended to disappear when the medium was enriched with insulin and/or cortisol (fig. 20-24, 42).

The results of the experiments are summarized in tables I and II, using a grading system from 0 to 6, with 0 representing total necrosis and 6 representing complete maintenance, i.e., the explant was essentially indistinguishable from the original tumor. Each explant was rated individually for degree of survival after completely studying all of the serial sections. The survival ratings appear in the column headed "Survival" in both tables. Fortunately, the range of response of well selected tumors to the various individual control and hormonal media was generally narrow and reproducible.

As previously mentioned, the control tissues (199 alone, 199 plus 0.8% or less ethanol, and 199 plus 0.3% ethanol plus 50 micrograms per ml. of cholesterol) were indistinguishable from each other, and closely resemble the original tumor except for the presence of a few karyolytic and pyknotic nuclei.

The explants cultured in medium containing 0.1 micrograms per ml. of testosterone (fig. 14) showed no significant morphological difference from the controls (fig. 11-13) (table I). With as little as 1.0 micrograms per ml. of testosterone in the medium, definite morphologic changes were found in explants from older tumors after one day in culture (fig. 15). These consisted mainly of an increased number of nuclei showing pyknosis, karyolysis, and karyorrhexis. Karyolysis was more frequently observed than pyknosis. With 10 to 50 micrograms per ml. of testosterone in the medium, there was a definite increase in the number of degenerating and necrotic cells, regardless of whether the explant came from a young or old tumor (fig. 16,17). Explants from older tumors showed slightly more degeneration and necrosis, resulting in a wider range of explant survival. Also, the central areas of explants and of tumor cell nests within explants were frequently the first areas to show degeneration. The neoplastic epithelial cells attached to the basement membranes of the acini on the periphery of the tumor were the last to die. Concentrations of testosterone of 50 micrograms per ml. or greater consistently produced total degenerative change and necrosis after 2 1/2 to 3 days in culture (fig. 17-19 (table I).

The effect of testosterone on tumor explants was modified in part by the addition to the medium of insulin and/or cortisol (table II). Whereas 50 micrograms per ml. of testosterone

produced complete necrosis at 3 days (fig. 17), addition of 50 to 100 micrograms per ml. of amorphous insulin resulted in survival of explants at the 4 (fig. 26) to 6 (fig. 27) level (see grading system in Materials and Methods). Combinations of 200 or 5 or 1 micrograms per ml. of insulin with 50 micrograms per ml. of testosterone resulted in survival at the 0 to 2 level (fig. 28-30). Combination of 50 or 5 micrograms per ml. of insulin with 100 micrograms per ml. of testosterone resulted in survival at the 0 to 1 level (fig. 31, 32). Combinations of 100 or 50 micrograms per ml. of cortisol with 10 micrograms per ml. of testosterone resulted in survival at the 5 to 6 level after 3 days (fig. 33, 34), in contrast to survival at the 2 to 3 level after 3 days in medium containing 50 micrograms per ml. of testosterone alone. Combination of 10 micrograms per ml. of cortisol with 10 micrograms per ml. of testosterone resulted in survival at the 4 to 5 level (fig. 35) (table II). Combination of 100 micrograms per ml. of cortisol with 50 micrograms per ml. of testosterone resulted in survival at the 3 to 4 level after 3 days (fig. 36) in contrast to complete necrosis of the explant occurring after three days in medium containing 50 micrograms per ml. of testosterone alone. Combination of smaller amounts of cortisol (50 or 100 micrograms per ml.) with 50 micrograms per ml. of testosterone resulted in no significant enhancement of survival (fig. 37, 38). Explants

maintained in concentrations of cortisol from 1 to 50 micrograms per ml. survived at the 5 to 6 level after 3 days (fig. 22-24) (table II). Explants grown in 100 micrograms per ml. of cortisol alone showed survival at the 4 to 5 level after 3 days (fig. 25) in contrast to control tissues which survived at the 5 to 6 level.

Explants maintained in medium containing insulin, cortisol, and testosterone in concentrations of 100, 25, and 1 (or 10) micrograms per ml., respectively, survived at the 6 level (fig. 39, 40) (table II). However, explants cultured in medium containing insulin, cortisol, and testosterone in concentrations of 100, 25, and 100 micrograms per ml., respectively, were completely necrotic after 3 days (fig. 41) (table II).

In general, the simultaneous addition of insulin and cortisol (or to a slightly lesser extent, either hormone alone) to the culture medium resulted in a more healthy appearance of the neoplastic cells at the end of the culture period. This was manifested by a lack of the slight nuclear and cytoplasmic changes previously mentioned as occurring in the control explants or in explants maintained in 199 plus low concentrations of testosterone. The tissues maintained in 199 plus insulin plus cortisol closely resembled the original cells, with epithelial adherance to the basement membrane, maintenance of acinar pattern, absence of nuclear and cytoplasmic swelling, and overall persistence of cytoplasmic and nuclear characteristics of the original tumors. Karyolysis was

moderately prominent in cells maintained in medium 199 containing high concentrations of testosterone plus 50 micrograms per ml. of cortisol (fig. 36, 37).

The process of cytolysis in the testosterone enriched cultures and in control cultures of explants from old tumors were preceeded by a variety of advanced degenerative changes in the cells. These changes appeared to vary from cell to cell, and included nuclear swelling, nuclear pyknosis, karyolysis, karyorrhexis, increased cytoplasmic granularity, prominence of the nucleolus, and peripheral clumping of chromatin at the nuclear membrane. Following cytolysis, fragments of granular cytoplasmic and basophilic nuclear debris remained.

It is interesting to note that in concentrations of testosterone of 10 to 50 micrograms per ml., both alone and combined with insulin or cortisol (in concentrations which partially enhanced explant survival), the less degenerate cells occurred in clumps distributed throughout the explants with intervening areas of complete cell necrosis (e.g. fig. 16, 17A, 28, 32). In the higher concentrations of testosterone (50 to 300 micrograms per ml.) the entire explant appeared to be undergoing degeneration and necrosis simultaneously (e.g. fig. 17B, 18, 19, 41). In this circumstance most of the cells were in the same stage of degeneration and necrosis at one time, except for persistence of occasional peripheral tumor cells,

as previously mentioned.

In general, the fibrous tissue component of the explants was well maintained in both the control cultures and in the cultures containing low concentrations of testosterone. The addition of insulin and/or hydrocortisone to the medium did not affect the morphology of the stromal cells significantly. High concentrations of testosterone (those which produced total necrosis of the epithelial cells) also produced necrosis of the stromal connective tissue elements. Variations in the amount of connective tissue in the explants did not appear to significantly alter the effect of added hormones on the epithelial cells.

Electron Microscopy Portions of the mouse mammary adenocarcinomas were taken for electron microscopy at the time when the organ cultures were prepared (fig. 44-46). The ultrastructure of these tumors conformed to previous descriptions (9,10). The cells were often pyramidal in shape and arranged about a central lumen (fig. 44). Microvilli were frequently present at the luminal cell surface. Nuclei were often irregular in outline and displayed uneven distribution of electron densities. Mitochondria were rod-shaped with numerous cristae. Only minimal amounts of membranous ergastoplasm were present in most cells. The numerous ribonucleoprotein (RNP) particles usually occurred singly or in small groups. The Golgi apparatus was inconspicuous and without evidence of secretory droplet formation. As previously recorded, numerous virus-like particles were observed in many of the cells and occasionally, in extra cellular positions (fig. 44-46). Their morphology was similar to previous descriptions (15).

Control explants (fig. 47-52), maintained in 199 alone for 1 to 3 days closely resembled the original tumors by electron microscopy. Occasional degenerating cells were present (fig. 47). Some cells possessed a variety of cytoplasmic inclusions of variable morphology (fig. 48, 52). Virus-like particles similar to those observed in the original tumors were frequently noted (fig. 48, 51).

The tissue maintained for 3 days in medium 199 containing 50 micrograms per ml. of testosterone consisted largely of

of unrecognizable debris (fig. 53, 54). Nuclei, when still recognizable, showed irregular outlines, increase in density, and irregular chromatin clumps. Fragments of dense material were interpreted as remnants of karyorrhectic nuclei. Swollen, ovoid mitochondria were observed. Many of these contained internal bodies of irregular outline, high density, and unknown composition (fig. 53). Their density and homogeneity suggested that they might be fat; however, their location within degenerating mitochondria suggest that they may be remnants of cristae or altered mitochondrial matrix.

The addition of 100 micrograms of insulin to the medium containing 50 micrograms per ml. of testosterone partially protected the cells against the necrotizing autolytic effects of testosterone after 3 days of culture (fig. 55-57). A number of cells interpreted as necrotic and degenerating were present, however. The intact cells closely resembled those in the original tumors and in the control cultures. Occasional cells showed groups of ergastoplasmic sacs that were more extensive in amount than any seen in the original tumors or in the control cultures (fig. 57).

Tissue was studied after one day in media containing 0.1, 10, 100 and 300 micrograms per ml. of testosterone in order to search for early degenerative changes which might suggest sites of action of testosterone (fig. 58-66).

Epithelial cells which remained for one day in medium 199 with 0.1 micrograms per ml. of testosterone (fig. 58-60) were indistinguishable from cells of the original tumors and from the controls maintained in medium 199 alone. Numerous virus-like particles were observed (fig. 58, 60).

Cells in media with 10, 100 or 300 micrograms per ml. of testosterone showed patchy zones of cytolysis and necrosis intermingled with groups of intact, apparently unaltered cells (fig. 61-65). As in other preparations, virus-like particles were numerous and of the usual morphology (fig. 62). Cells with nuclei in various stages of karyorrhexis were observed in the specimens cultured in 100 or 300 micrograms per ml. of testosterone (fig. 65). Irregularly-shaped nuclei with coarse clumping of their internal constituents were frequently observed (fig. 61, 63). Cytoplasm of cytolyzed cells was generally reduced to largely unrecognizable disorganized masses of granular and vacuolar debris (fig. 61, 63-65). Occasional cells interpreted as in the process of early degeneration showed swollen mitochondria and relatively empty cytoplasmic vacuoles (fig. 66). The prevalence of the mitochondrial swelling, disrupted cristae, and of dense intra-mitochondrial granules, previously mentioned (fig. 53), suggested that one of the important sites of direct or indirect action of testosterone might be in these organelles, known to be the major localization of the respiratory enzymes.

Discussion: Several factors concerning the methodology of these experiments should be considered. A completely chemically defined synthetic maintenance medium was used, thus avoiding all of the complicating unknown factors in complex, protein-enriched media. That the medium itself had little or no toxic effect on C3H mouse mammary adenocarcinoma explants is demonstrated not only by the extensive controls included in this experiment but also by the work of others (30, 74, *).

It might be suggested that the experiments should have utilized complex biologic media on the basis that such media might be more physiologic. However, according to Elias and Rivera (30) explants of C3H mouse mammary adenocarcinoma in organ culture responded in a similar manner to both medium 199 alone and to complex biologic media containing chick serum and chick embryo extract. Further, this response was not altered by the addition, to either 199 alone or to the biologic media, of various combinations and concentrations of estrogen, progesterone, cortisol, somatotropin, and mammotropin, unlike normal prelactating mammary tissue and hyperplastic alveolar nodules. Lasfargues (60) observed that chicken serum does not favor the growth of mouse mammary epithelium, which may explain the lack of response of C3H mouse mammary adenocarcinoma

*Rivera, E. Personal communication, 1963.

explants to the biologic media used by Elias and Rivera. Lasfargues further found that human cord serum gave the longest survival of normal mouse mammary epithelium, and postulated that this finding was a result of the high concentrations of hormones in the cord serum. Indeed, even if human cord serum does prolong maintenance or cause growth of mouse mammary adenocarcinoma explants in organ culture, it certainly could not be considered a "physiologic" medium. It has been shown that neoplastic mouse mammary cells can survive at least 10 days in medium 199 alone, and that inoculation of the cultured explant into the donor strain gives rise to tumors histologically similar to the original tumor (74). These facts, considered together, demonstrate that medium 199 is a suitable medium in which to conduct the present experiments.

The nature and effect of the minute amounts of tissue fluid in the explants plus substances derived from degenerating cells was an uncontrollable variable in these experiments. The amount of extracellular fluid present in explants was minimized by using tumors with very little stroma and by washing explants thoroughly in medium 199 before culturing them. Cells with completely normal fine structural characteristics were found in immediate contact with completely necrotic cells. This fact supports the view that at least the degenerating cells did

not release toxic substances.

The histologic variability of the tumor itself presented no problem of interpretation of results as long as healthy, small tumors were used. These small tumors had a uniform architecture and consisted almost entirely of epithelial cells with minimal stroma. It seems possible, however, that the tumors differed intrinsically in metabolic or growth characteristics not detectible by histological examination. However, the uniform histological detail of the selected tumors, the fact that none metastasized, and the consistent pattern of response of the explants to the various treatments used in this experiment, all suggest the essential similarity of the several different tumors used.

Although the purest available hormones were added to the culture medium, the possible presence of trace amounts of active impurities cannot be excluded or affirmed. It seems improbable that the well-defined pattern of apparently dose-related response to testosterone and the other hormones used, singly and in combination, would have been obtained if significant impurities were present.

The growth inhibitory effect of testosterone on various normal and neoplastic tissues has been demonstrated previously. Stone (100) has observed almost 100% inhibition of growth of

HeLa cells *in vitro* by as little as 5 micrograms per ml. of testosterone. These results are consistent with the direct toxic effect of testosterone demonstrated in the present experiments.

It is possible that a portion of the testosterone was in micro-crystalline form, rather than in solution, under the conditions used in the present experiments. However, Algard (6) has shown distinct, measurable effects of micro-crystalline mixtures of diethylstilbesterol and testosterone on neoplastic mouse epithelium *in vitro* in concentrations as low as 5 micrograms per ml. Chemically related hormones with similar solubility characteristics have been shown to be active *in vitro* (28, 29, 30, 74, 88, 89, 90, 91, *). Further, in these experiments, the steroid nucleus (cholesterol) and a steroid hormone (cortisol), both closely related structurally to testosterone, caused no significant toxic effect. Indeed, cortisol at the concentrations used helped to maintain the morphologic characteristics of the tumor explants. In one experiment in the present series insulin and cortisol (100 and 12 micrograms per ml., respectively) resulted in excellent explant survival (table II). After the addition of estrogen (0.6 micrograms per ml.) alone or estrogen

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*Rivera, E. Personal communication, 1963.

and progesterone (0.6 micrograms per ml. each) together to the medium containing insulin and cortisol, the explants not only showed excellent survival but, in addition, their acinar lumens were filled with dense, eosinophilic material of a possible secretory nature. Although this particular experiment was not repeated, these results help support the contention that, under the conditions of these experiments, hormones were affecting cellular metabolism. This result also hints that some C3H mouse mammary adenocarcinomata may be able (perhaps because of a high degree of differentiation) to respond physiologically to hormones. Supporting this idea is the observation that cell suspensions of trypsinized spontaneous and transplanted C3H mouse mammary adenocarcinomata, cultured in vitro in complex media, can reassociate and form gland-like structures with secretions in their lumens (81). Also, tumors in lactating mammary glands of rats frequently contain milk or colostrum. Fibroadenomatous tumor transplants in pregnant rats frequently develop into pure adenomas with fat globules and other secretions in their lumens (46).

It is tempting to speculate on the manner in which testosterone may produce cell death. In these experiments the tumor cells which cytolyzed in the medium containing effective concentrations of testosterone, often showed unusual electron dense bodies in

their mitochondria. As far as is known these bodies have not been previously noted, at least among the commonly occurring degenerative changes (18, 75, 107). In addition, in a large majority of degenerating cells in the testosterone-enriched medium, the mitochondria were swollen, and their cristae were fragmented. These mitochondrial changes are commonly considered to be non-specific and degenerative in nature (75). The mitochondria in the degenerating cells of the control cultures showed similar, but much less marked, changes. These observations suggest that the mitochondria may be a site of direct or indirect action of testosterone. There is much evidence for a deficiency of electron transport systems in tumors which predisposes them to cellular metabolic respiratory failure. Tumor cells also exhibit a respiratory-glycolytic imbalance. They may obtain 100 times as much energy from fermentative processes (aerobic and anaerobic glycolysis) as from respiration, in comparison to the reverse picture in normal tissues (58, 105). It has been postulated that a relative incapacity of enzymes in the tumor cell mitochondria would cause failure of catobolism of the pyruvate produced by the excessive fermentation (58). Perhaps testosterone enhances this relative incapacity of the tumor cell to catabolize pyruvate, or lactic acid, or other substances present resulting in accumulations so great as to

cause deleterious effects within the cell. The ability of testosterone to markedly inhibit pyruvate metabolism by the citric acid cycle enzymes of isolated liver mitochondria has been shown (40).

Another route by which testosterone may fatally effect tumor cell metabolism at the level of the mitochondria is by interfering with their already deficient electron transport system. Green (41) has indicated that the electron transport system of cells is intimately related to their mitochondria. Huggins (51, 52, 53) has shown that the ability of testosterone derivatives to inhibit tumor growth is closely related to their molecular structure and that strong electron donors or electron receptors are generally most effective. Perhaps testosterone enhances a pre-existing deficiency in electron transport in the mitochondria of the tumor cell, causing metabolic or energetic failure.

In spite of the observation that insulin has no measurable effect on glucose utilization by mouse mammary tumor explants (74), it has been shown to enhance mitochondrial glycolysis in other tumors (50). Cortisone and corticosterone have been observed to increase citrate synthesis in mitochondria (40). These two findings may correlate with the improved cytomorphologic appearance of mouse mammary adenocarcinoma cells cultured in medium containing insulin and/or cortisol and may

also explain the apparent protective action of insulin and/or cortisol demonstrated in these experiments.

The degenerating cells observed by light and electron microscopy showed many of the structural changes generally associated with non-specific degeneration (18, 75, 97). The unknown inclusion bodies (e.g. fig. 48) found in the cytoplasm of some of the tissues observed by electron microscopy may correspond to altered mitochondria described by Morgan et. al. (75) as occurring in degenerating cells. The unusual inclusions seen in figure 52 closely resemble the "myelin bodies" mentioned by DeMan and van Rijssel (22). The process of cytolysis of epithelial cells with release of free nuclei, mitochondria, and other structures compares with the involutional cytomorphological changes described by Wellings and DeOme as occurring in the post-lactational mammary gland of normal mice (107). Indeed, the previously discussed mitochondrial changes were the only unusual ones noted. Except for these changes, it can be concluded that no distinct morphologic pathway of degeneration exists for mammary cancer cells treated with testosterone under the conditions of this experiment.

The nonspecific effect described in the cells cultured in low concentrations of testosterone or in the control media may

have been a result of an unfavorable pH of the medium occurring at some time during the culture period (18), in spite of careful adjustment of the gaseous flow rate and other precautions taken. Or it may have been due to slight cellular injury resulting from the lack of some substance (or of the ability to utilize it) which was partially compensated for by the addition of insulin and/or hydrocortisone. That testosterone (and similarly, insulin and/or hydrocortisone) have effects on individual cells is supported by observations made in these experiments. Apparently perfectly healthy cells, as judged morphologically by their fine structure, were seen to exist contiguous to remnants of once adjacent cells, apparently completely destroyed by the effects of testosterone.

The significance of experimental work with mouse mammary tumors in understanding the factors that influence mammary cancer in the human have been discussed by Bittner (16). Knowledge of mouse mammary carcinoma is insufficient to be of much clinical use, and men differ from mice in many ways. However, the Fell technique of organ culture has recently been thoroughly studied along many parameters (55), and similar methods have been suggested as suitable for examining the problems of human tumor sensitivity to cancer chemotherapeutic agents (109). Investigations have already been started along these lines

(49, 57). The philosophy behind this research tool, its present status, and its bright future possibilities have been outlined by Fell (34).

Summary and Conclusions: In summary, it has been shown that:

1. In the presence of testosterone, C3H mouse mammary adenocarcinoma explants undergo total necrosis *in vitro* in a chemically defined medium, whereas controls do not.

2. The addition of insulin and/or cortisol to the chemically defined medium protects the explants from the toxic effects of testosterone.

3. The morphology of the tumor explants was best maintained by enriching the defined medium with insulin and/or cortisol.

4. The experimental procedures and media employed had no noticeable effect on the virus-like particles seen in electron micrographs of the tumor explants.

5. A direct toxic effect of testosterone on mammary adenocarcinoma of the mouse has been demonstrated.

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TABLE I

Survival of control and Testosterone treated explants of C3H mouse mammary carcinomas in organ culture

MEDIUM		TOTAL EXPLANTS 10 mice	SURVIVAL RATING 1-1 1/2 days	2 1/2-3 days
199, + ETOH (0.8% or less) + CHOL (50 in 0.3% ETOH)		103	5-6	5-6
199 + Test. (in maximum of 0.8% ETOH)	Test (γ)			
	0.1	13	5	5
	1.0	19	4-5	5
	10	28	3-4	2-3
	50	20	2	0
	100	29	1	0
	300	12	0	0

Note: gamma (γ) = micrograms/ml.

TABLE II

Effects of various hormones on survival of explants of C3H mouse mammary carcinoma after three days in organ culture

MEDIUM ( )	Total Explants	Survival Rating
I (100)	3	6
I (100) + CHOL (50)	8	5-6
I (100) + F (12)	3	6
I (100) + F (25)	8	6
I (100) + F (12) + E (0.6)	3	6
I (100) + F (12) + E (0.6) + P (0.6)	3	6
I (100) + F (25) + T (1)	10	6
I (100) + F (25) + T (10)	5	6
I (100) + F (25) + T (100)	8	0
I (200) + T (50)	4	2
I (100) + T (50)	8	5-6
I (50) + T (50)	4	4-5
I (5) + T (50)	4	1-2
I (1) + T (50)	4	1
I (50) + T (100)	4	0-1
I (5) + T (100)	4	0-1
F (100)	3	4-5
F (50)	4	5-6
F (10)	4	5-6
F (1)	5	5-6
F (100) + T (10	4	5-6
F (50) + T (10)	5	5
F (10) + T (10)	5	4-5
F (100) + T (50)	4	3-4
F (50) + T (50)	3	0-1
F (10) + T (50)	5	0-1

Note: 1) I= amorphous insulin, F= cortisol, E= estradiol-17 B, P= progesterone, CHOL= cholesterol, T= testosterone
2) Numbers in parentheses are concentrations in gamma ( ) or micrograms/ml. of preceeding hormones.

Figure 1.

A. Petri dish halves (9 cm. in diameter).

B. Sheets of Whatman No. 2 filter paper.

C. Plastic spacers.

D. Pyrex watch glass (6.0 cm. in diameter).

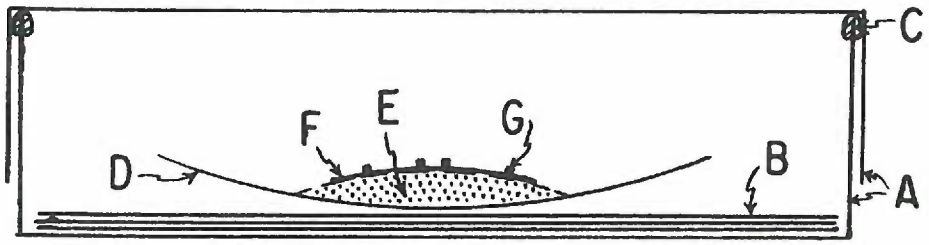
E. Culture medium (1 ml. quantity).

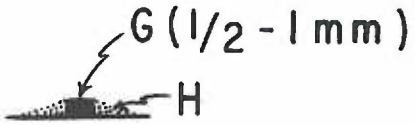
F. Siliconized lens paper float (2.2 cm. in diameter).

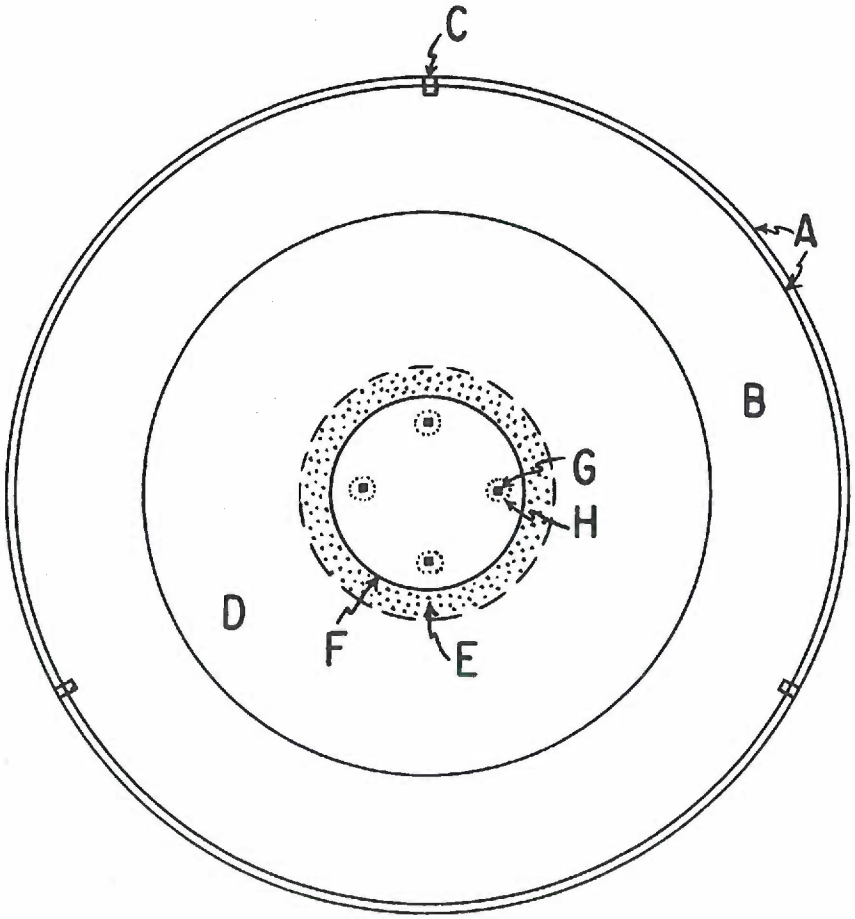
G. Tissue explant (1/2 - 1 mm. in diameter).

H. Meniscus of culture medium surrounding explant (see enlarged offset).

NOTE: The top and bottom figures are actual size.



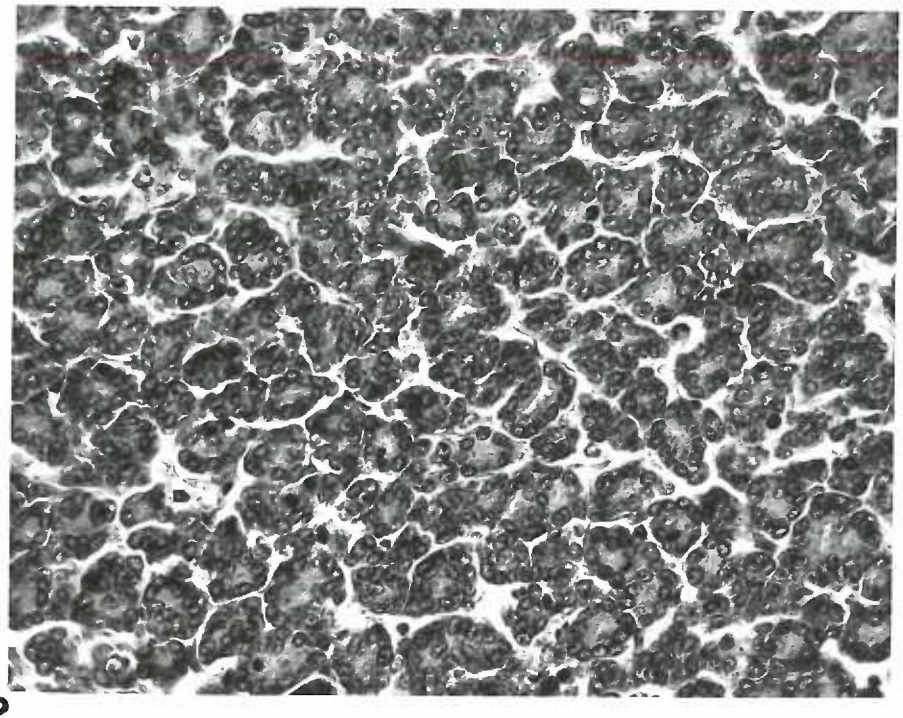




1

Figure 2. Mouse mammary adenocarcinoma. Original tumor, not cultured. Diameter of tumor 0.6 cm. Acinar pattern. 330X.

Figure 3. Mouse mammary adenocarcinoma. Original tumor, not cultured. Diameter of tumor 0.8 cm. Cyst-like pattern. 330X.



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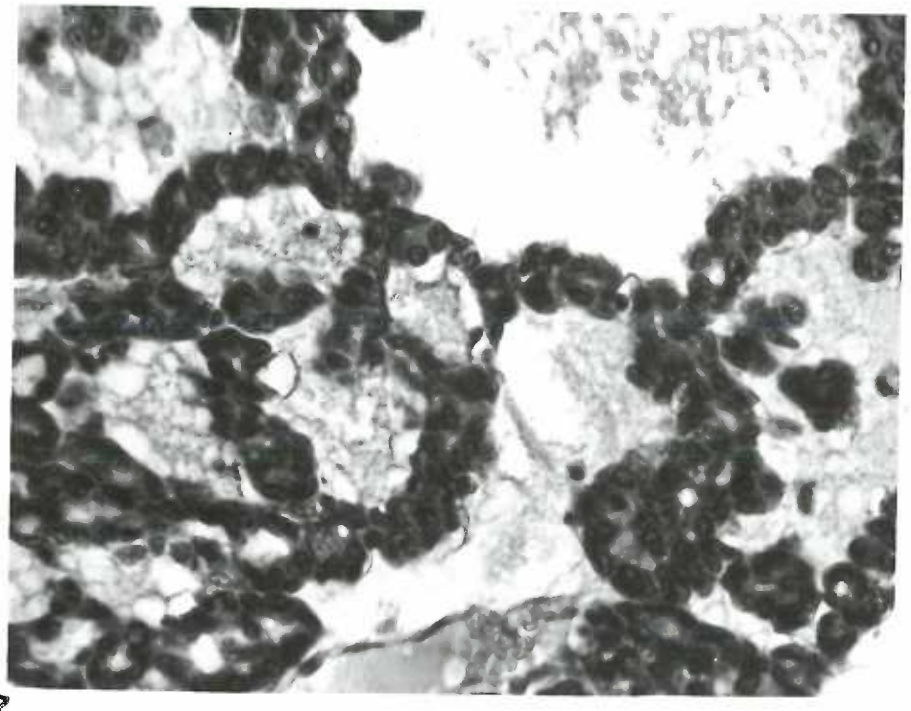
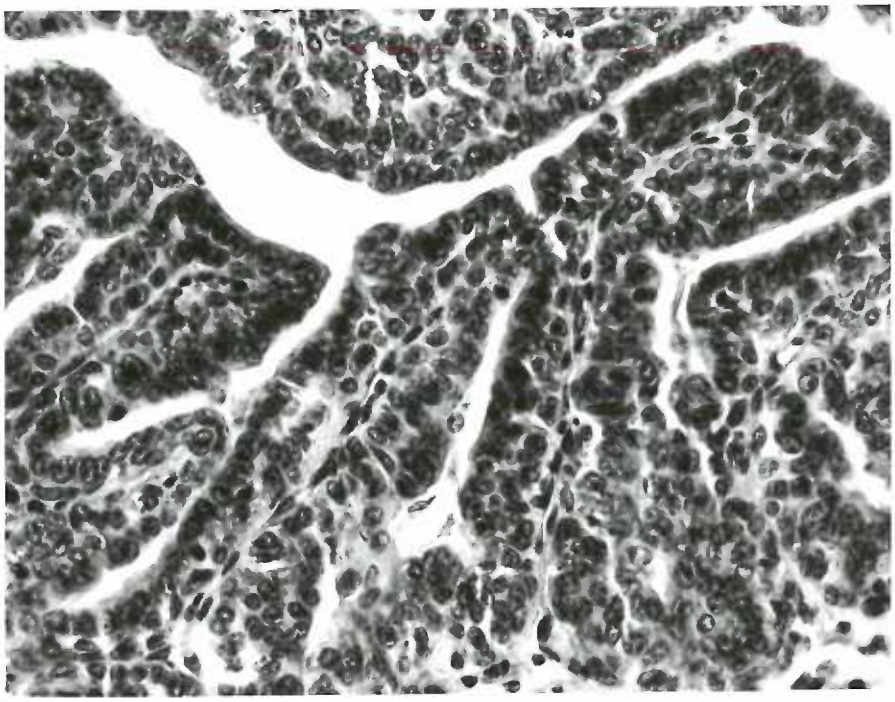


3

Figure 4. Mouse mammary adenocarcinoma. Original tumor, not cultured. Diameter of tumor 0.9 cm. Papillary pattern. 330X.

Figure 5. Mouse mammary adenocarcinoma. Original tumor, not cultured. Diameter of tumor 1.8 cm. Solid nests. (Note transitions from acini, to acini with piled-up epithelium, to solid nests in upper right). 330X.



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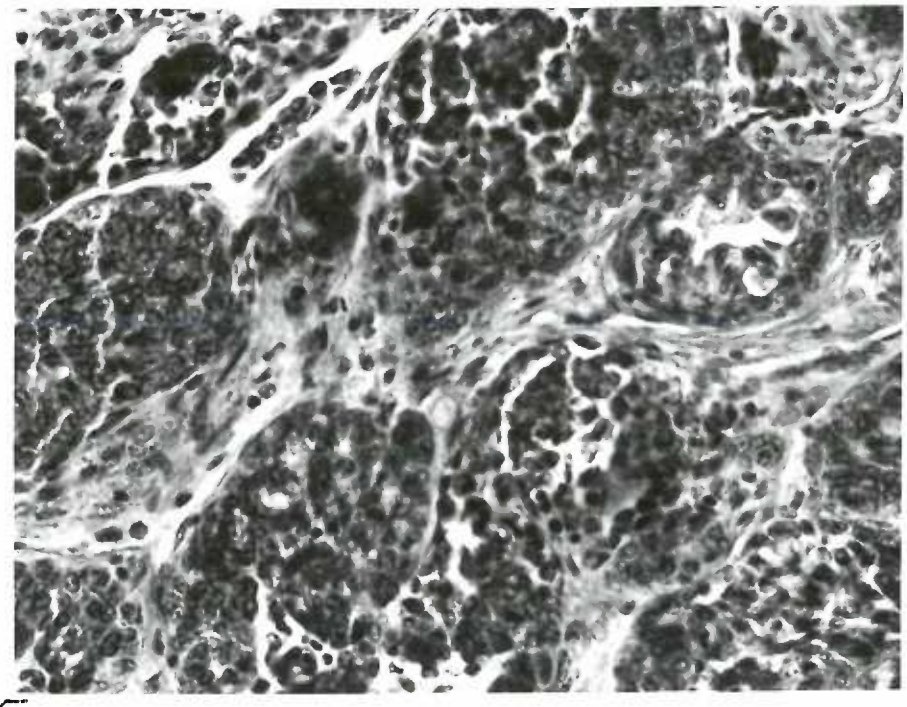

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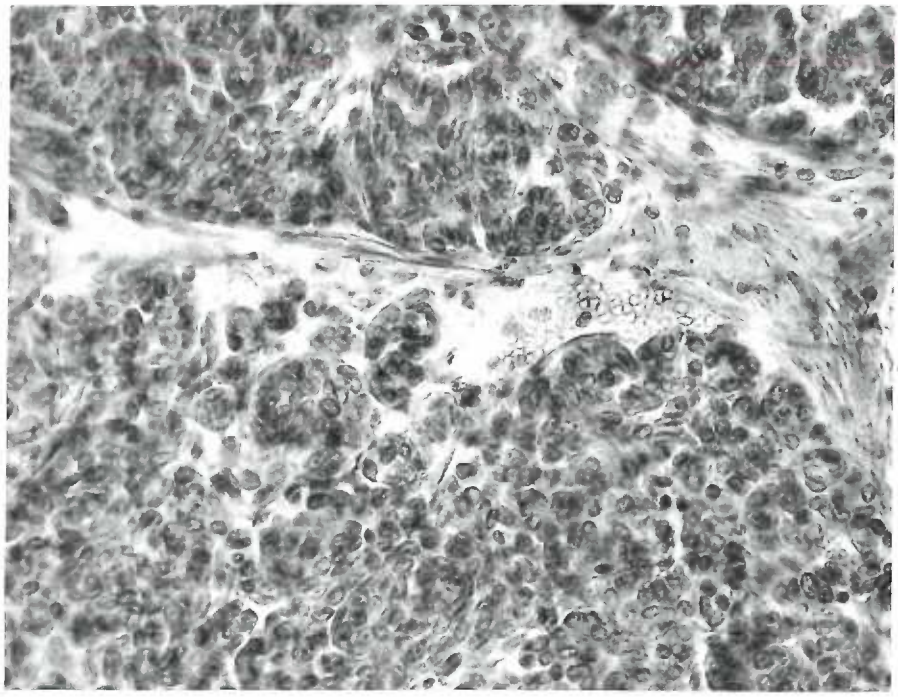
Figure 6. Mouse mammary adenocarcinoma. Original tumor, not cultured. Diameter of tumor 0.8 cm. Acini, solid nests, trabeculae. 330X.

Figure 7. Mouse mammary adenocarcinoma. Original tumor, not cultured. Diameter of tumor 2.0 cm. Old tumor with secretions and stromal fibrosis. Large acini. 330X.

Figure 8. Mouse mammary adenocarcinoma. Original tumor, not cultured. Diameter of tumor 1.3 cm. Sheet-like pattern with some stromal fibrosis. 330X.

Figure 9. Mouse mammary adenocarcinoma. Original tumor, not cultured. Diameter of tumor 1.7 cm. Old tumor with "pearl" formation (arrows), and nest-like pattern. 330X.



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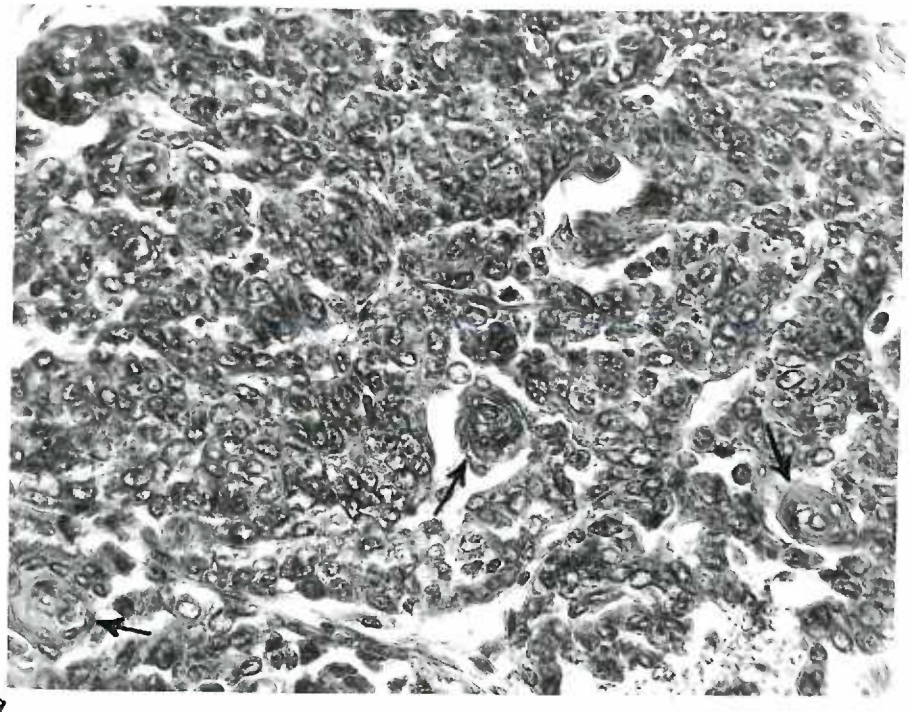
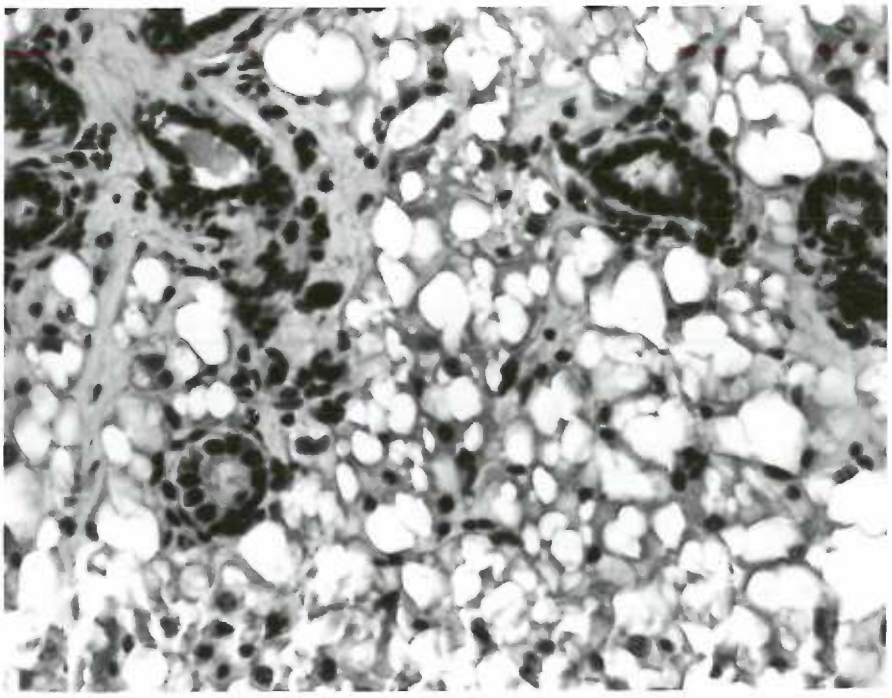


9

Figure 10. Normal resting mammary gland, old retired breeding female. Secretions in lumens. Fatty stroma. 330X.

Note: Subsequent figures 11 through 43 are of mouse mammary adenocarcinoma in organ culture.

Figure 11a. Cultured in medium 199 alone for three days. Note sharply delimited central necrosis of explant, and thin rim of peripheral necrosis (lower left). 135X.



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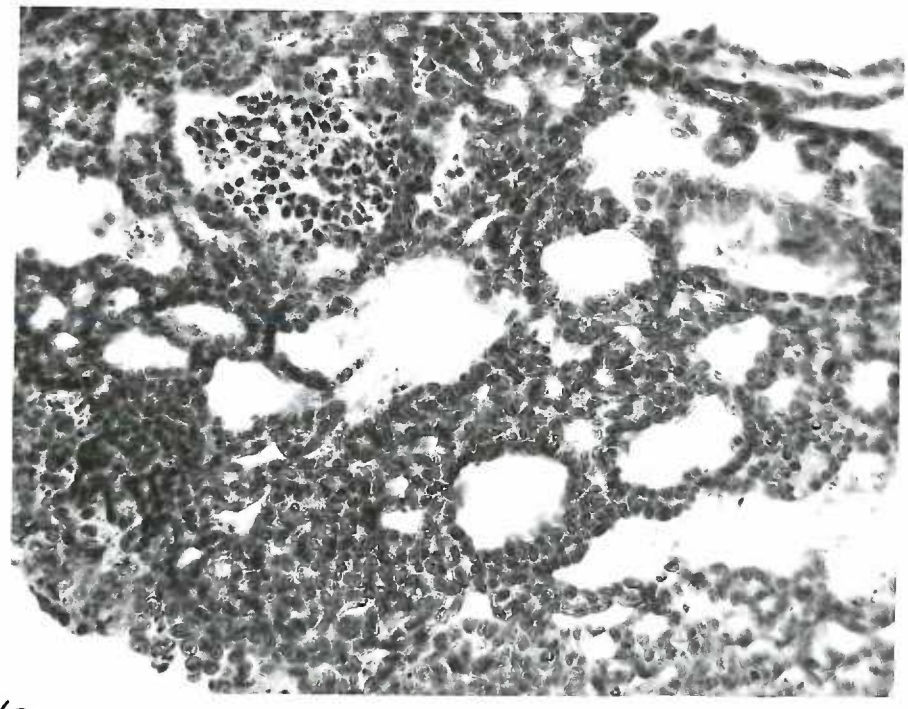
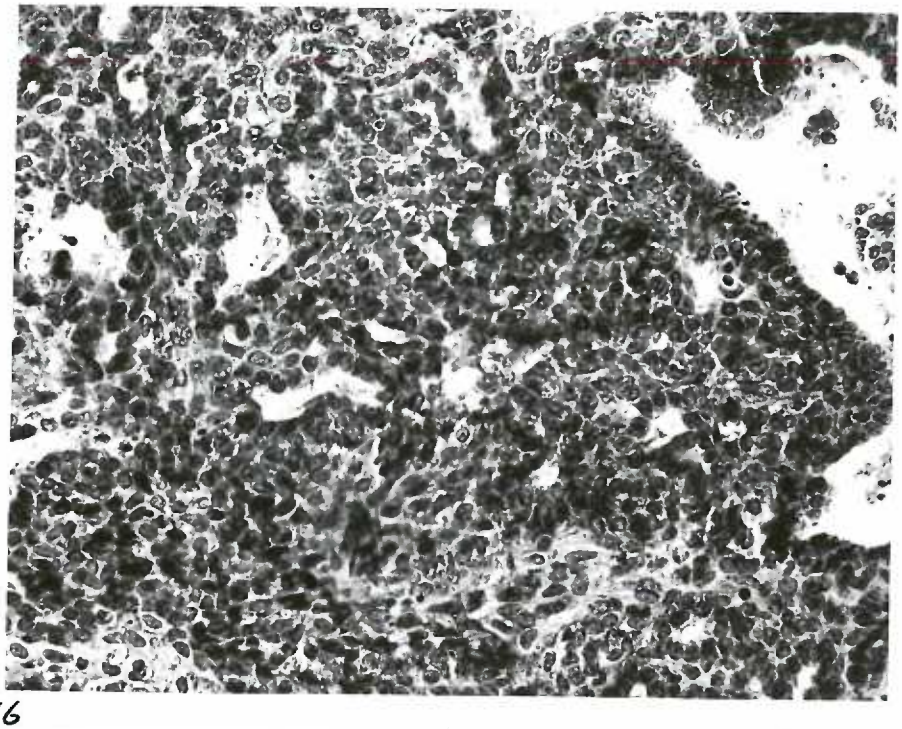


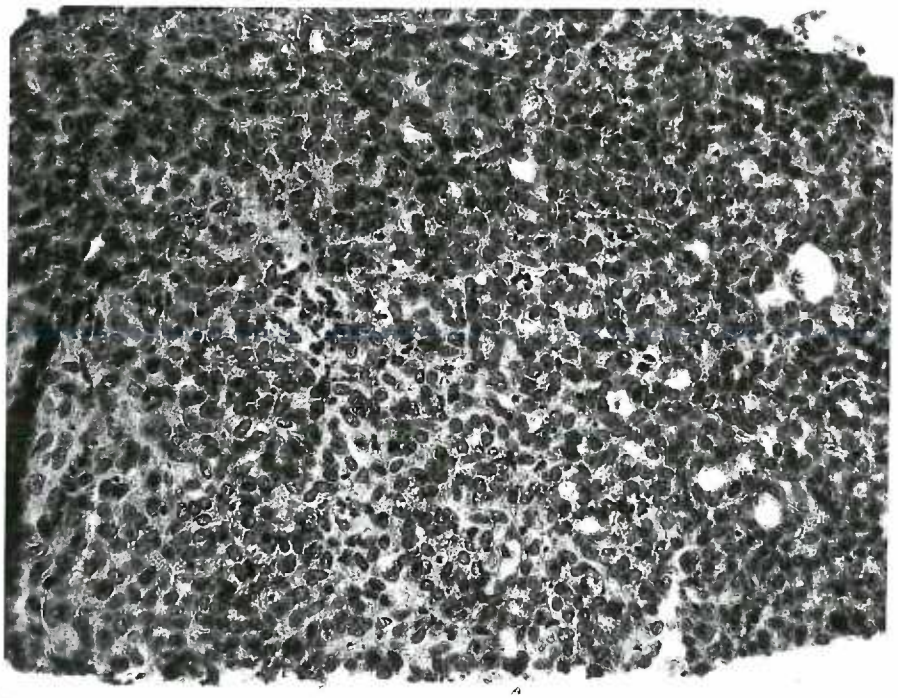
11a

Figure 11b. Cultured in medium 199 alone for three days. Note scattered pyknotic and karyolytic nuclei. 275X.

Figure 12a. Cultured in medium 199 with 0.8% ethanol for three days. 275X.



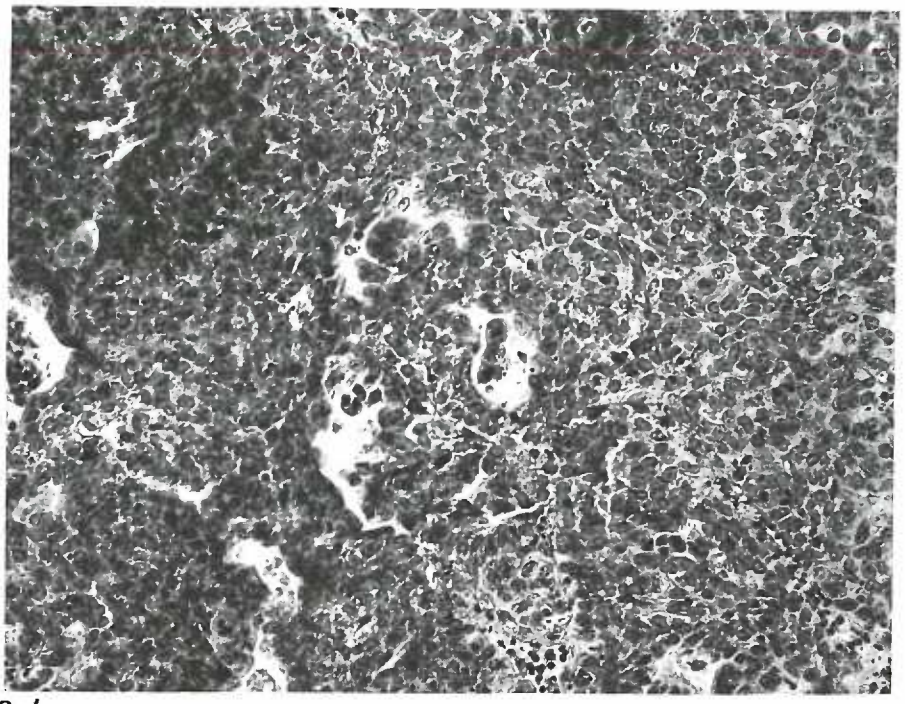
116



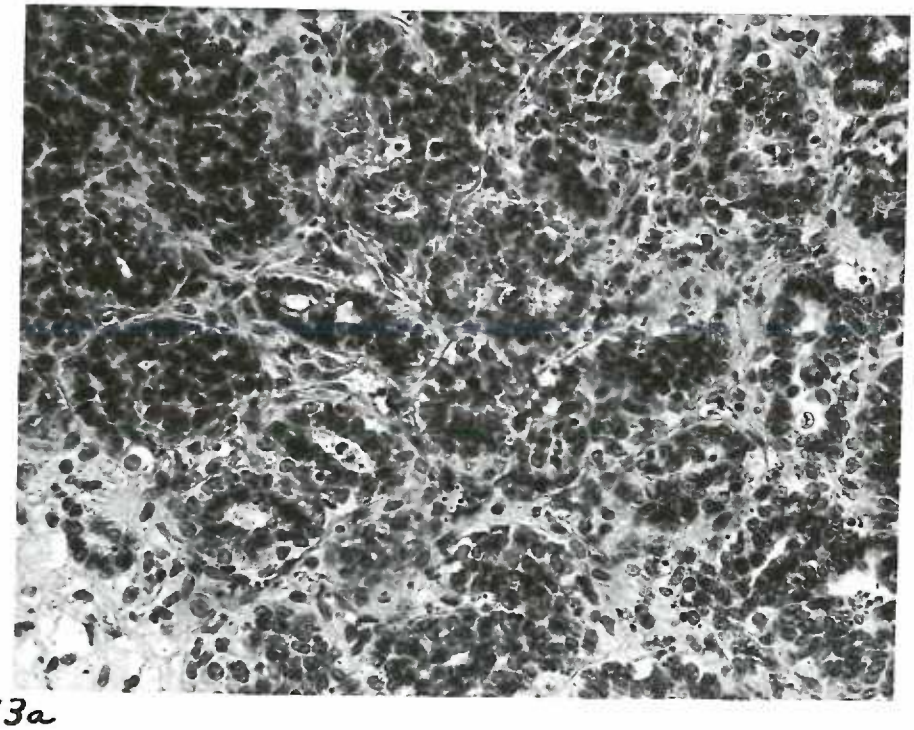
12a

Figure 12b. Cultured in medium 199 with 0.3% ethanol for three days. 275X.

Figure 13a. Cultured in medium 199 with 0.3% ethanol and 50 micrograms per ml. of cholesterol for three days. 275X.



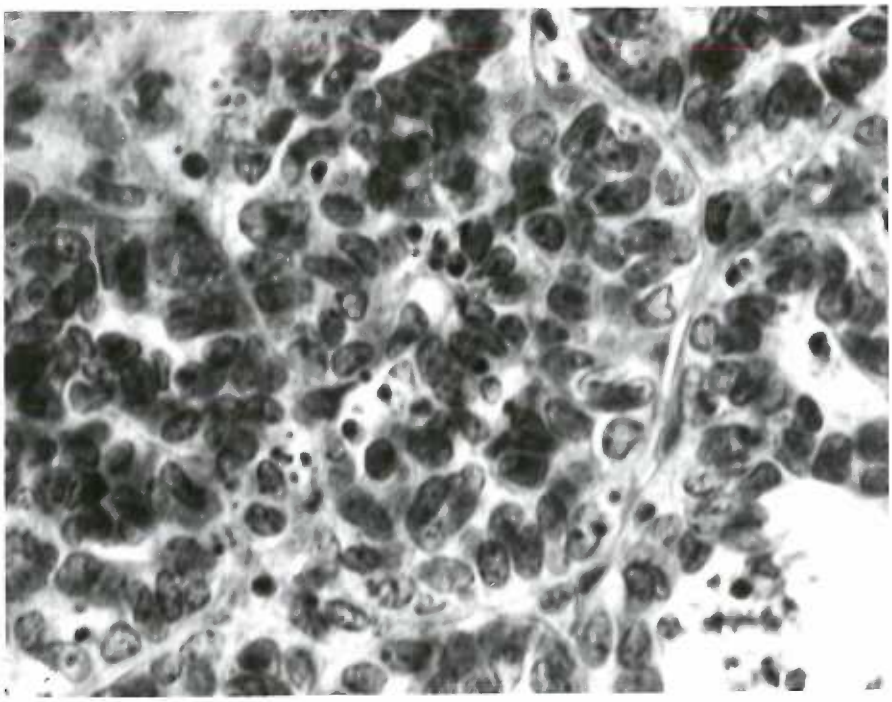
12b



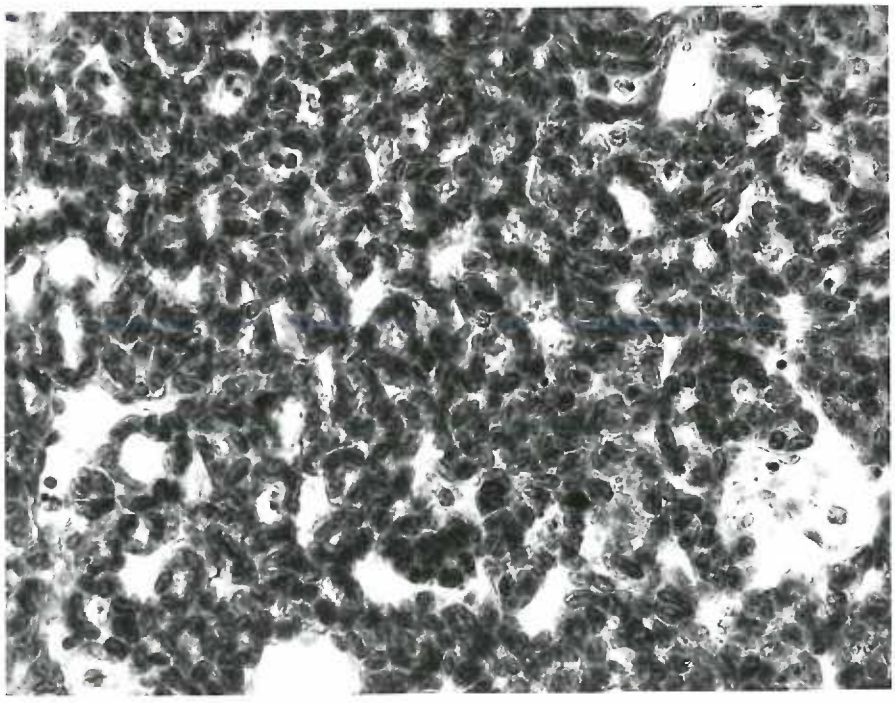
13a

Figure 13b. Same as 13a. 665X.

Figure 14. Cultured in medium 199 with 0.1 micrograms per ml. of testosterone for 3 days. 330X.



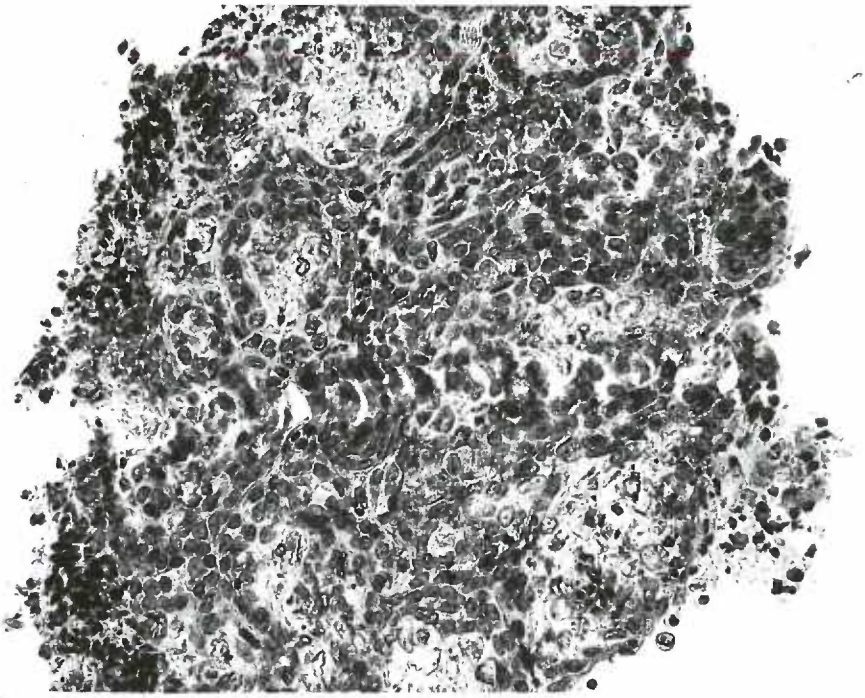
136



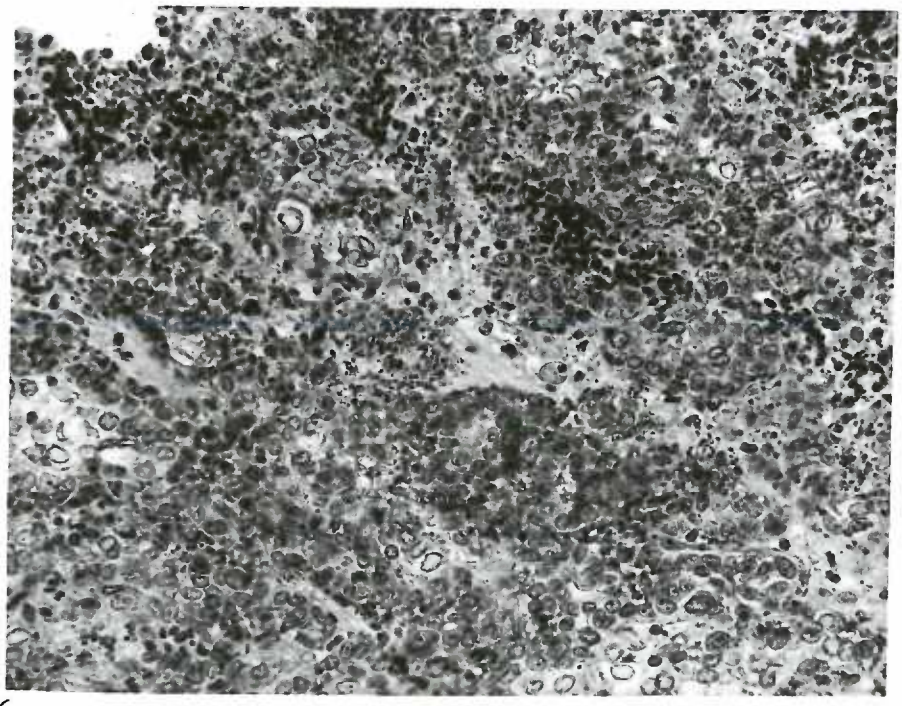
14

Figure 15. Cultured in medium 199 with 1.0 micrograms per ml. of testosterone for three days. 275X.

Figure 16. Cultured in medium 199 with 10 micrograms per ml. of testosterone for three days. 275X.



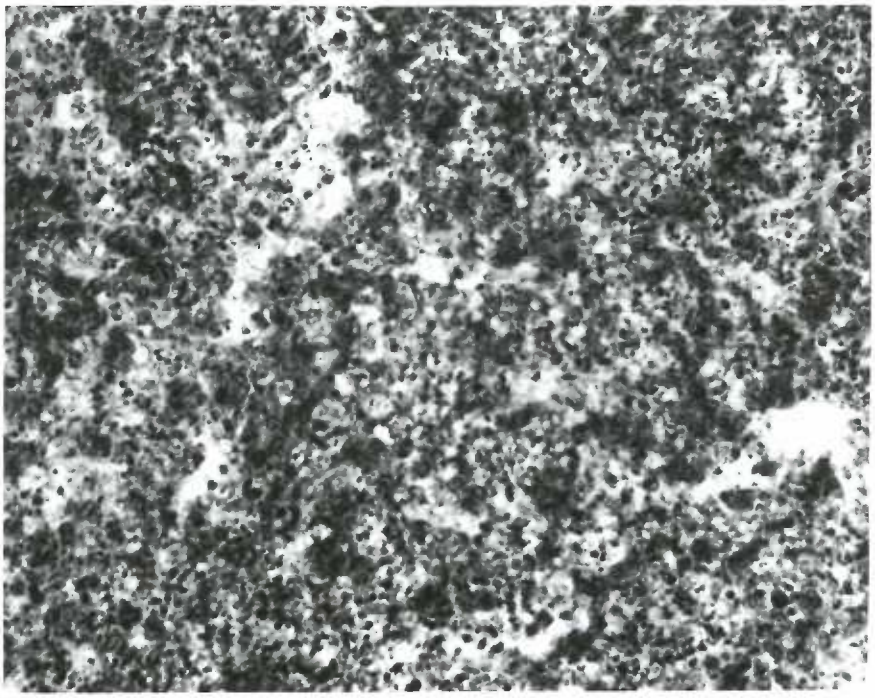
15



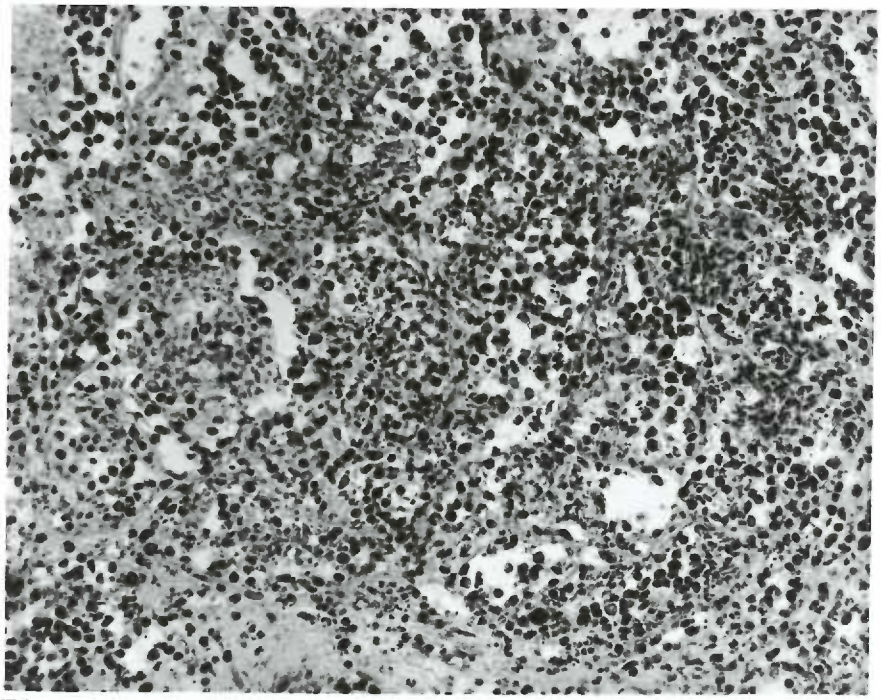
16

Figure 17a. Cultured in medium 199 with 50 micrograms of testosterone for three days. Young tumor with no stromal fibrosis and with clumps of viable-appearing cells. 275X.

Figure 17b. Cultured in medium 199 with 50 micrograms per ml. of testosterone for three days. Old tumor with some stromal fibrosis (bottom) and total necrosis. 275X.



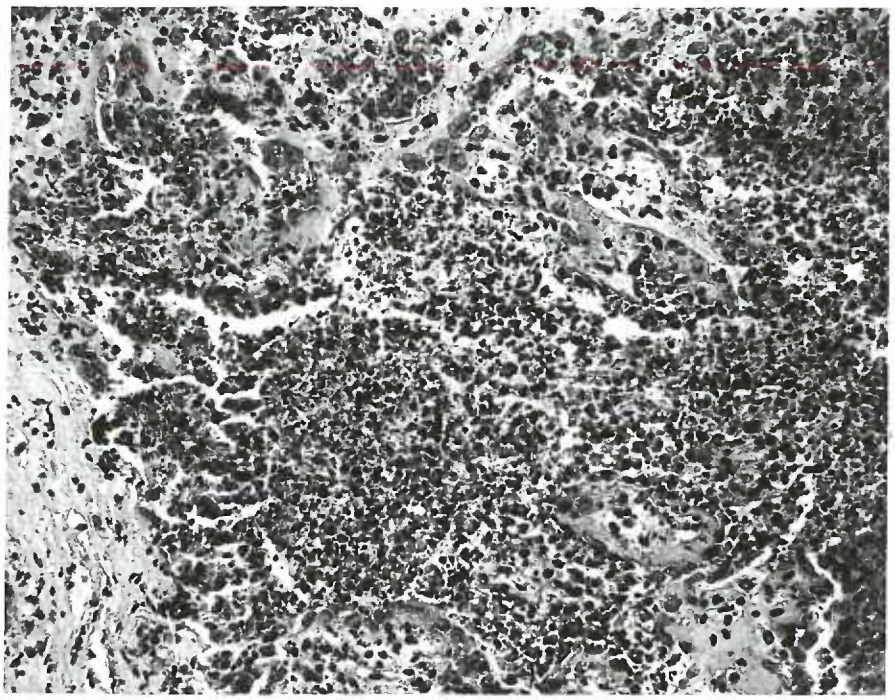
17a



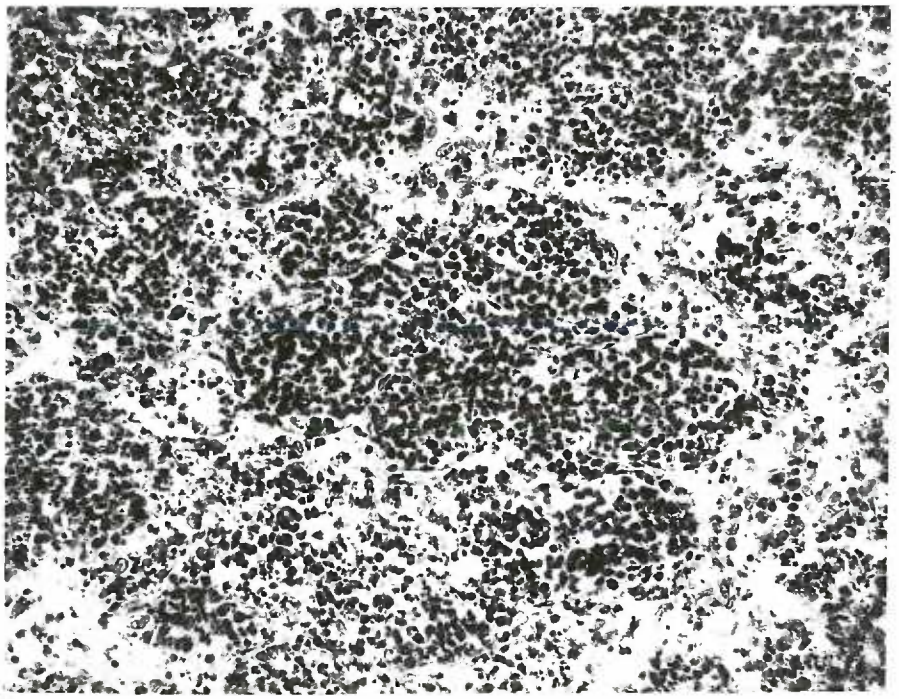
17b

Figure 18. Cultured in medium 199 with 100 micrograms per ml. of testosterone for three days. Note total necrosis, both epithelial and stromal cells.

Figure 19. Cultured in medium 199 with 300 micrograms per ml. of testosterone for three days.



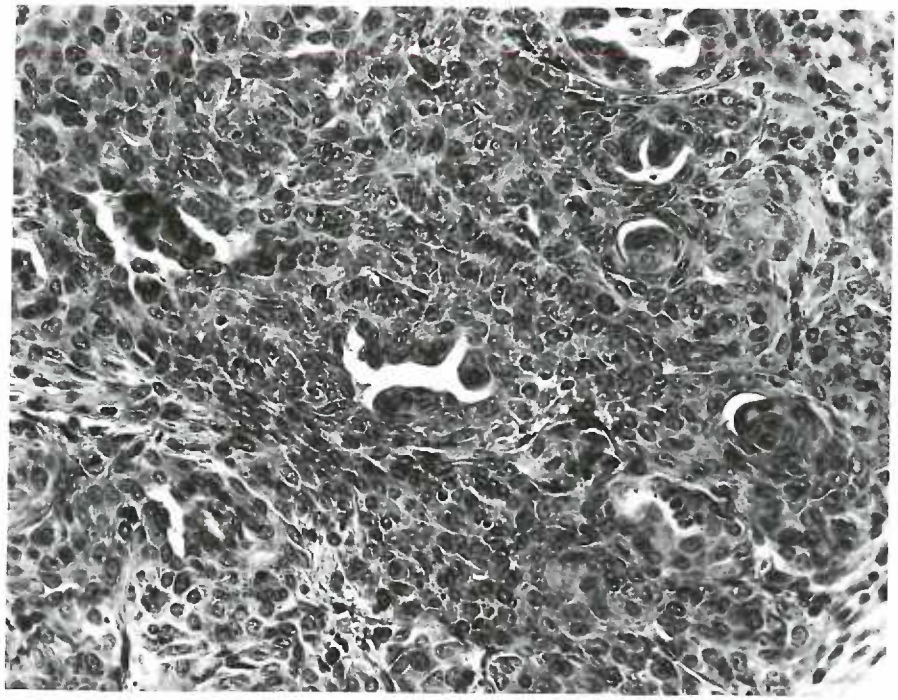
18



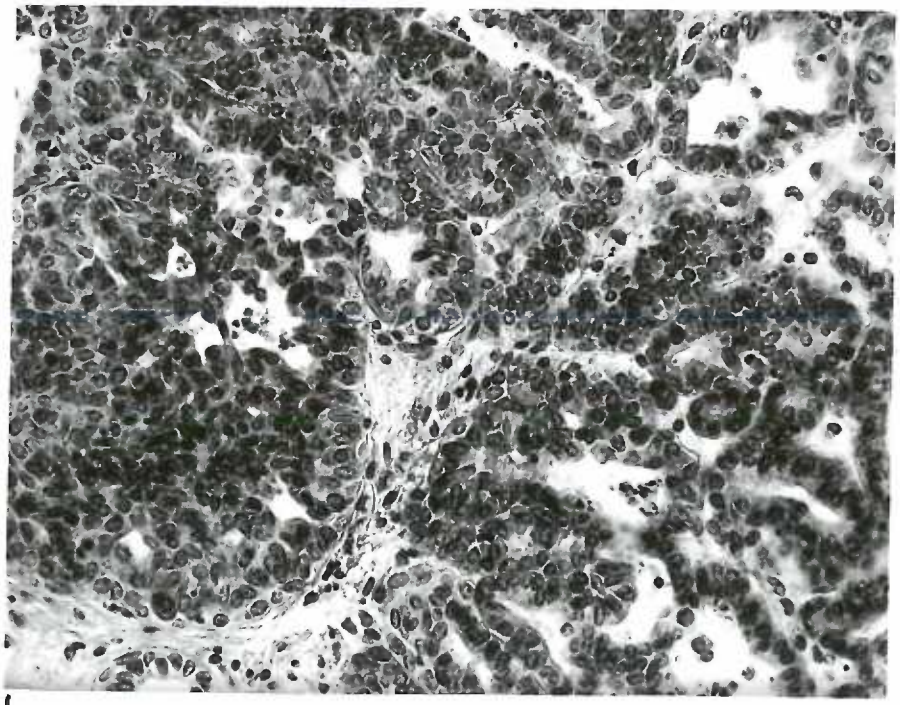
19

Figure 20. Cultured in medium 199 with 100 micrograms per ml. of insulin for three days. Note healthy appearance of cells. 275X.

Figure 21. Cultured in medium 199 with 100 micrograms per ml. of insulin and 50 micrograms per ml. of cholesterol for three days. 375X.



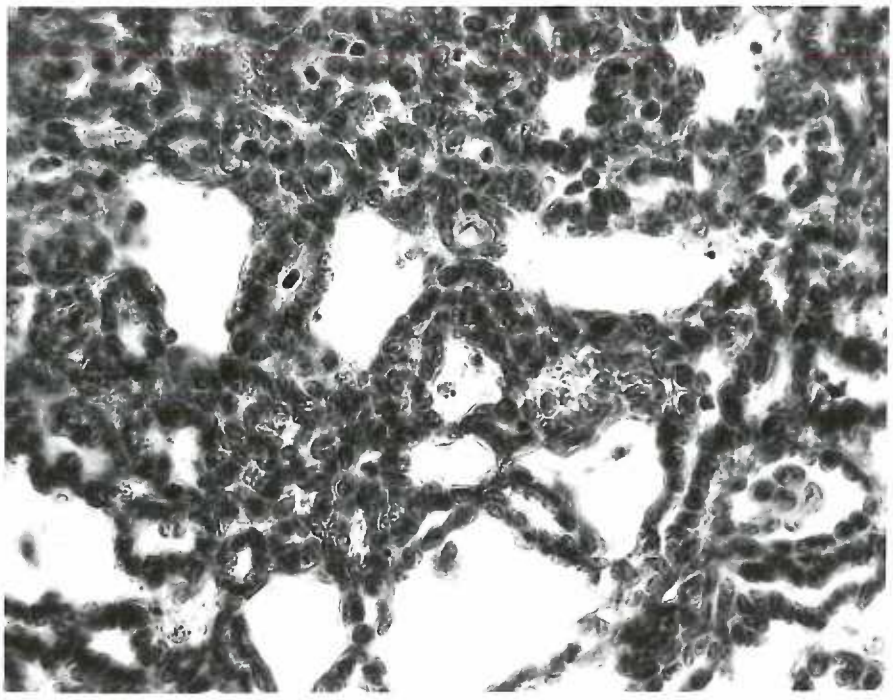
20



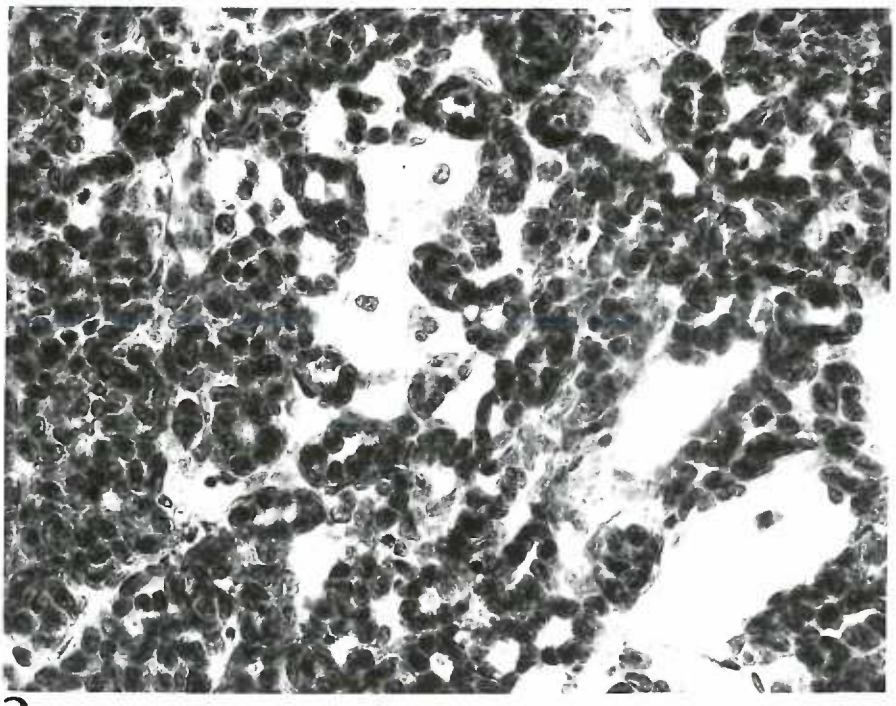
21

Figure 22. Cultured in medium 199 with 1.0 micrograms per ml. of cortisol for three days. 330X.

Figure 23. Cultured in medium 199 with 10 micrograms per ml. of cortisol for three days. 330X.



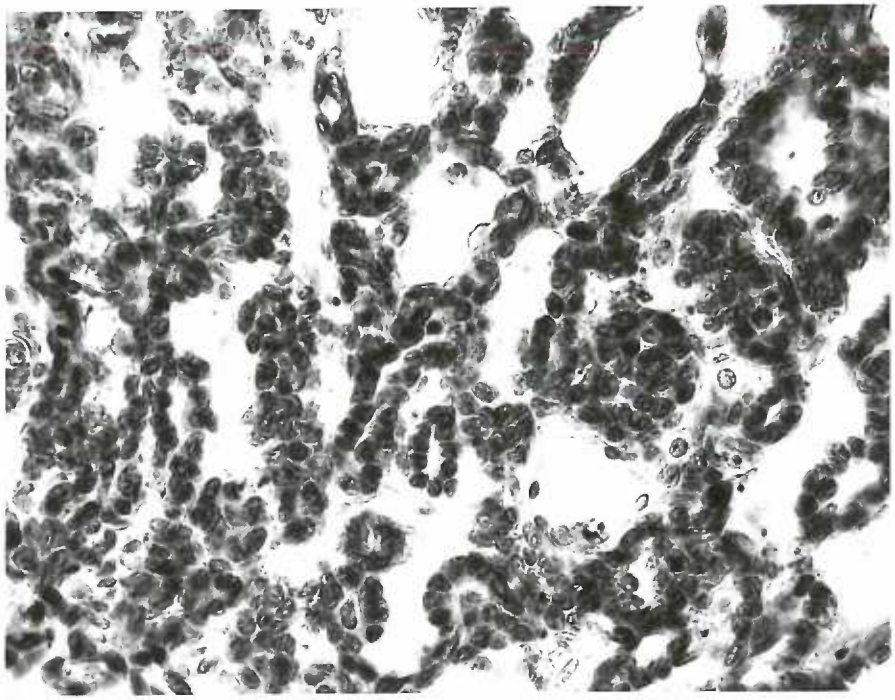
22



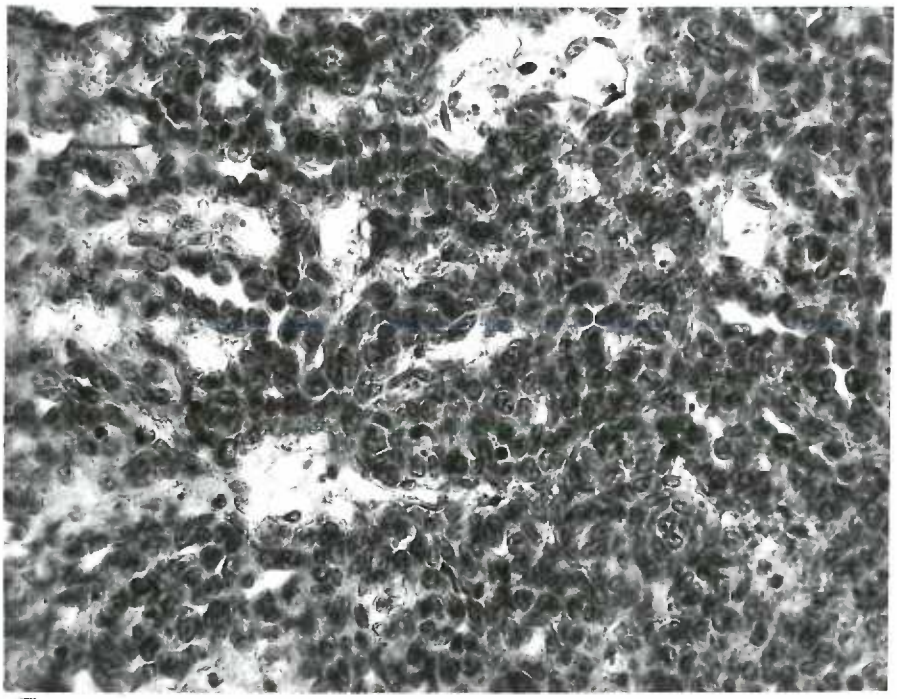
23

Figure 24. Cultured in medium 199 with 50 micrograms per ml. of cortisol for three days. 330X.

Figure 25. Cultured in medium 199 with 100 micrograms per ml. of cortisol for three days. 330X.



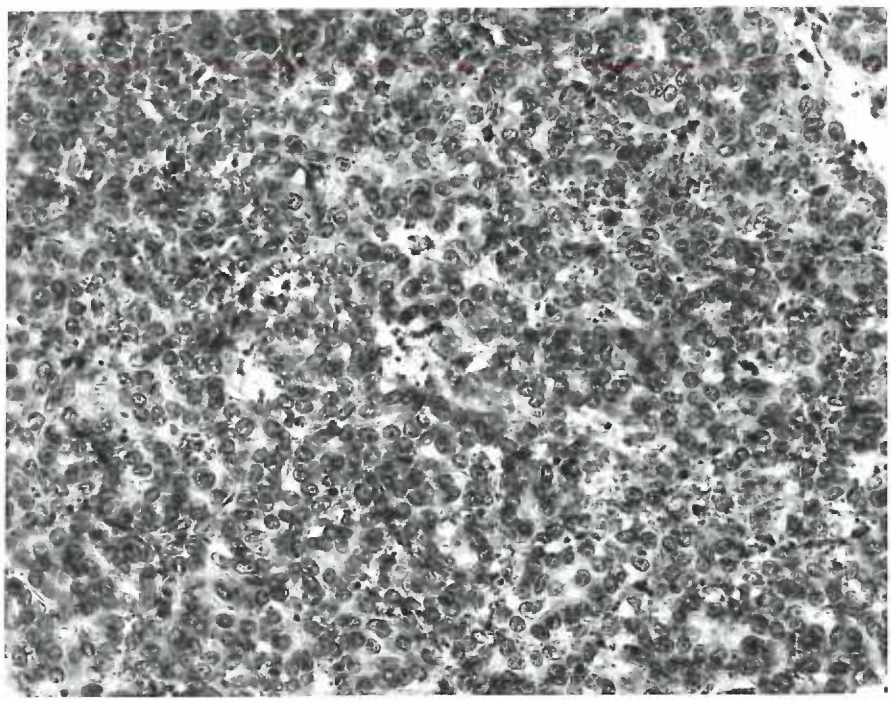
24



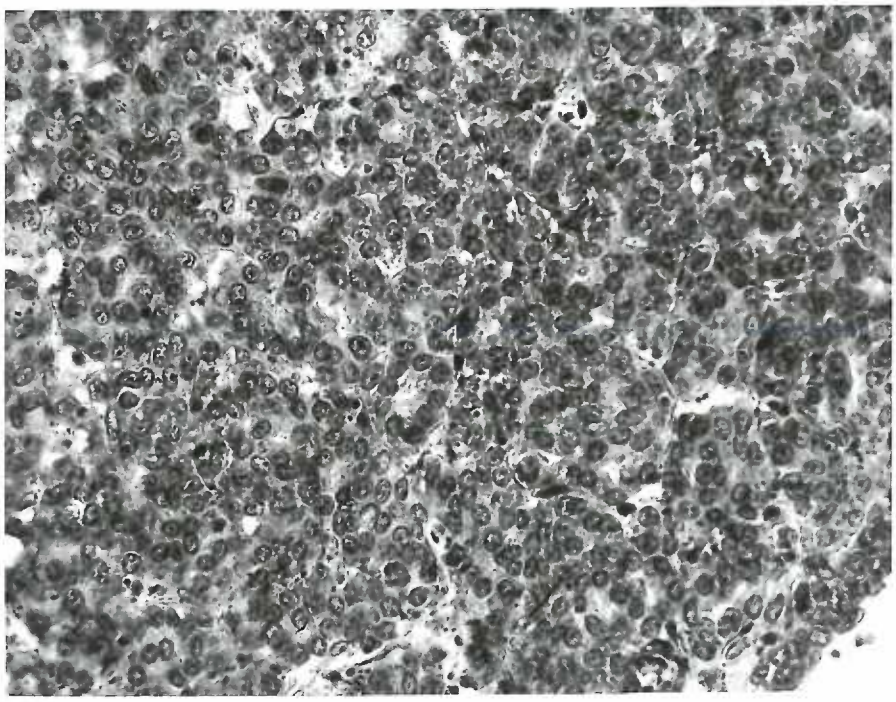
25

Figure 26a. Cultured in medium 199 with 50 micrograms per ml. of insulin and 50 micrograms per ml. of testosterone for three days. 330X.

Figure 26b. Same as 26a. 330X.



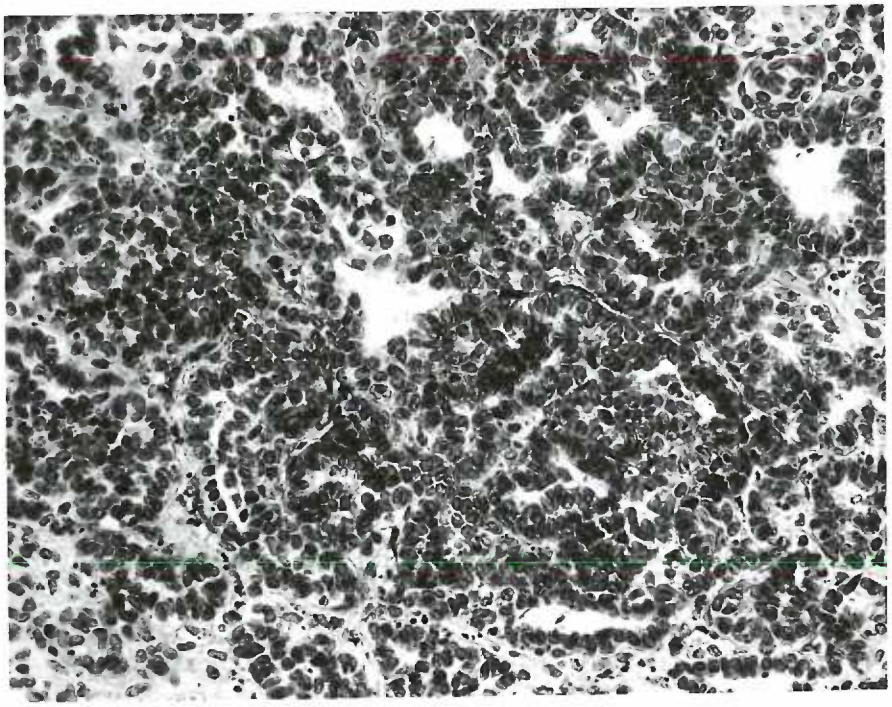
26a



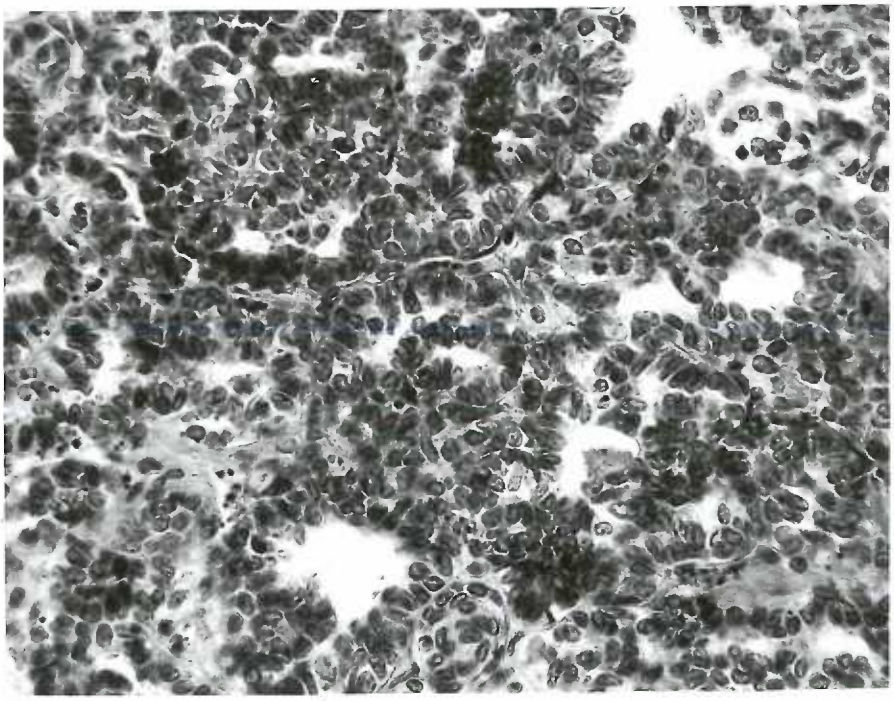
26b

Figure 27a. Cultured in medium 199 with 100 micrograms per ml. of insulin and 50 micrograms per ml. of testosterone for three days. 275X.

Figure 27b. Same as 27a. 330X.



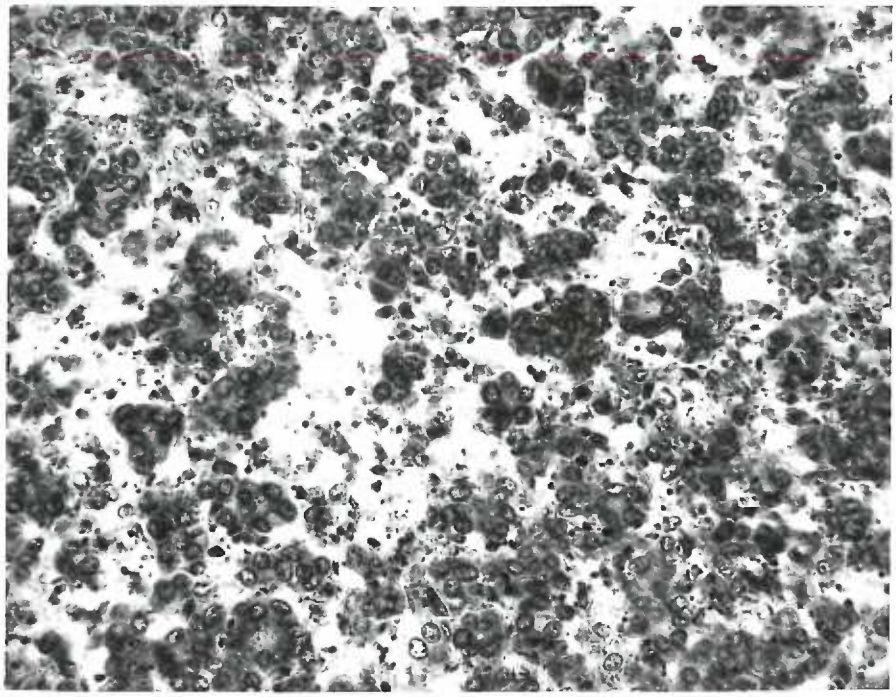
27a



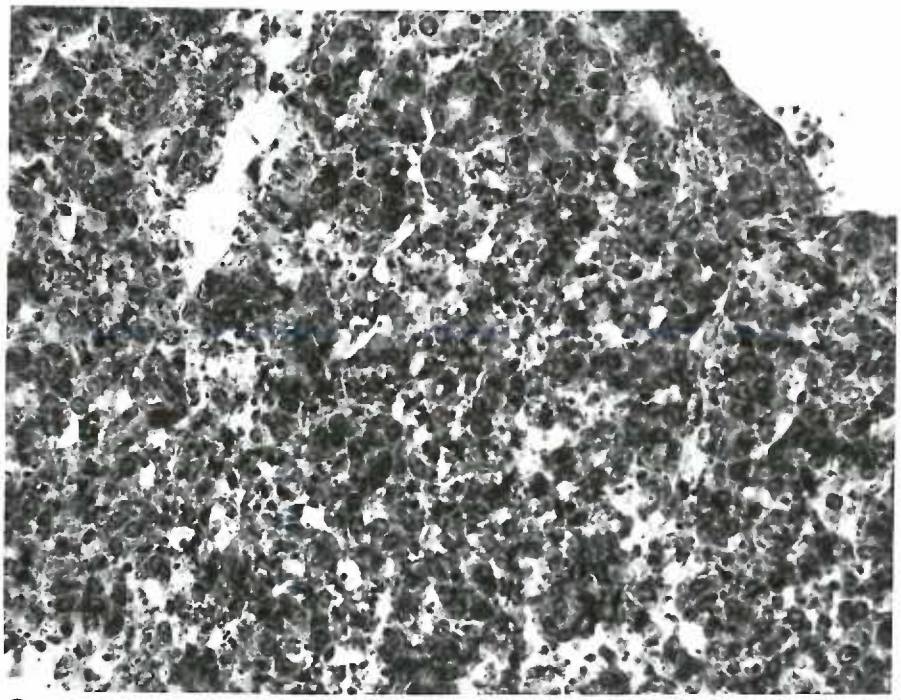
27b

Figure 28. Cultured in medium 199 with 200 micrograms per ml. of insulin and 50 micrograms per ml. of testosterone for three days. Note clumps of viable appearing cells. 330X.

Figure 29. Cultured in medium 199 with 5.0 micrograms per ml. of insulin and 50 micrograms per ml. of testosterone for three days. Note clumps of viable appearing cells. 330X.



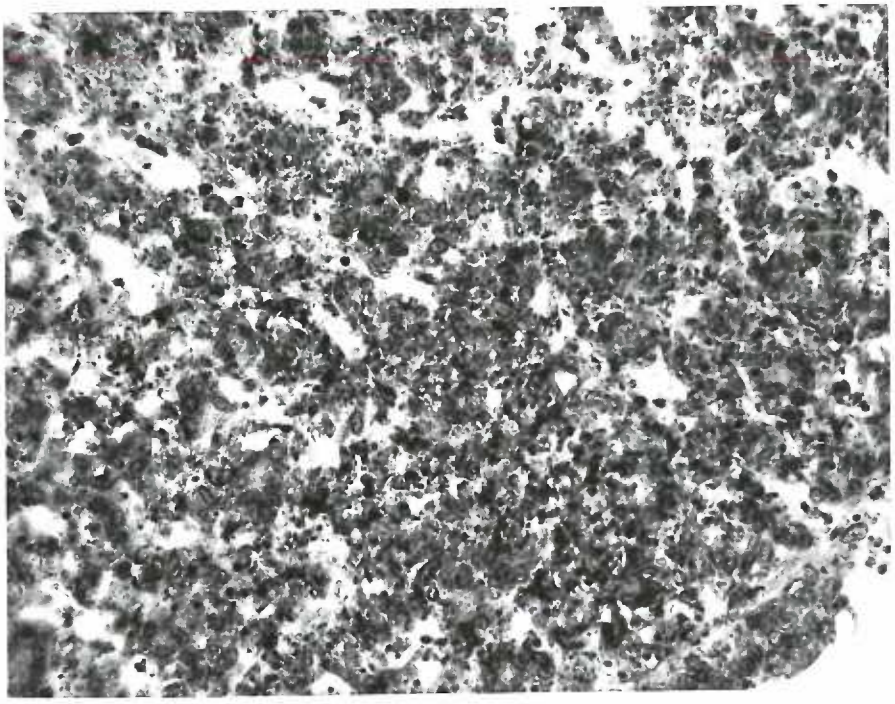
28



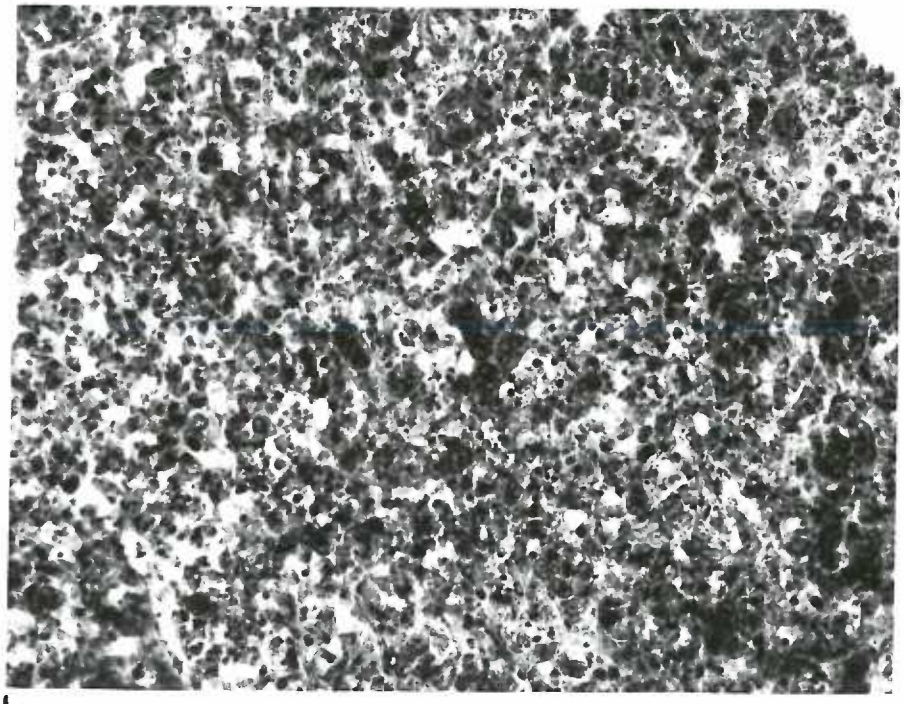
29

Figure 30. Cultured in medium 199 with 1.0 micrograms per ml. of insulin and 50 micrograms per ml. of testosterone for three days. 330X.

Figure 31. Cultured in medium 199 with 50 micrograms per ml. of insulin and 100 micrograms per ml. of testosterone for three days. 330X.



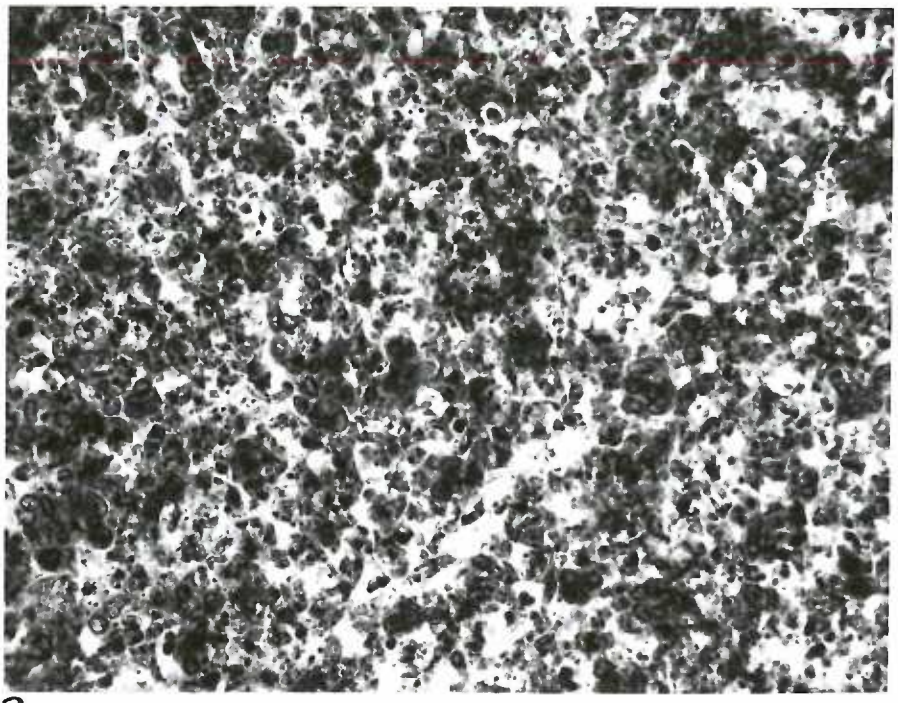
30



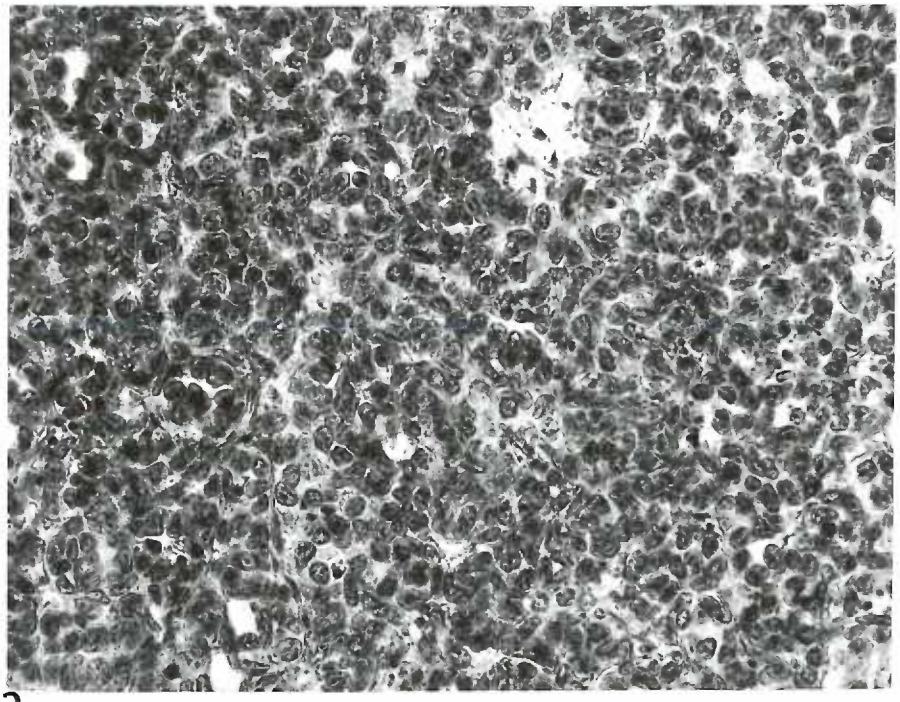
31

Figure 32. Cultured in medium 199 with 5.0 micrograms per ml. of insulin and 100 micrograms per ml. of testosterone for three days. 330X.

Figure 33. Cultured in medium 199 with 100 micrograms per ml. of cortisol and 10 micrograms per ml. of testosterone for three days. 330X.



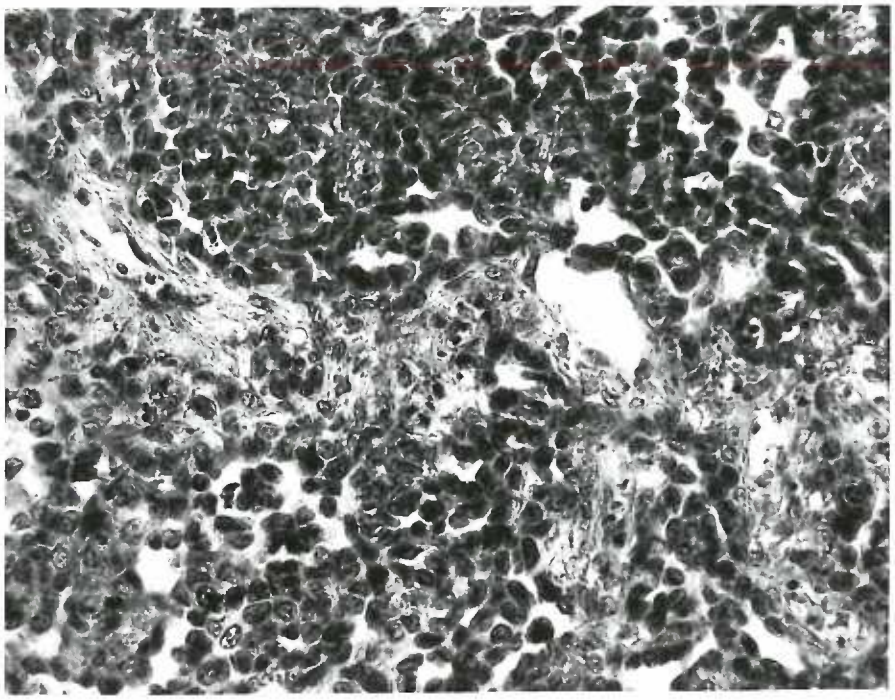
32



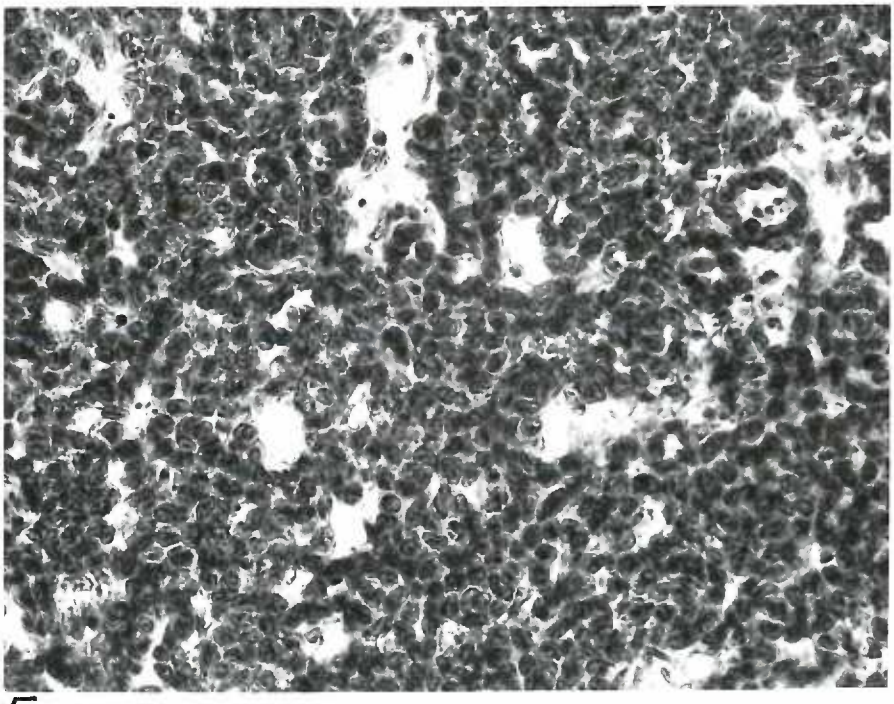
33

Figure 34. Cultured in medium 199 with 50 micrograms per ml. of cortisol and 10 micrograms per ml. of testosterone for three days. 330X.

Figure 35. Cultured in medium 199 with 10 micrograms per ml. of cortisol and 10 micrograms per ml. of testosterone for three days. 330X.



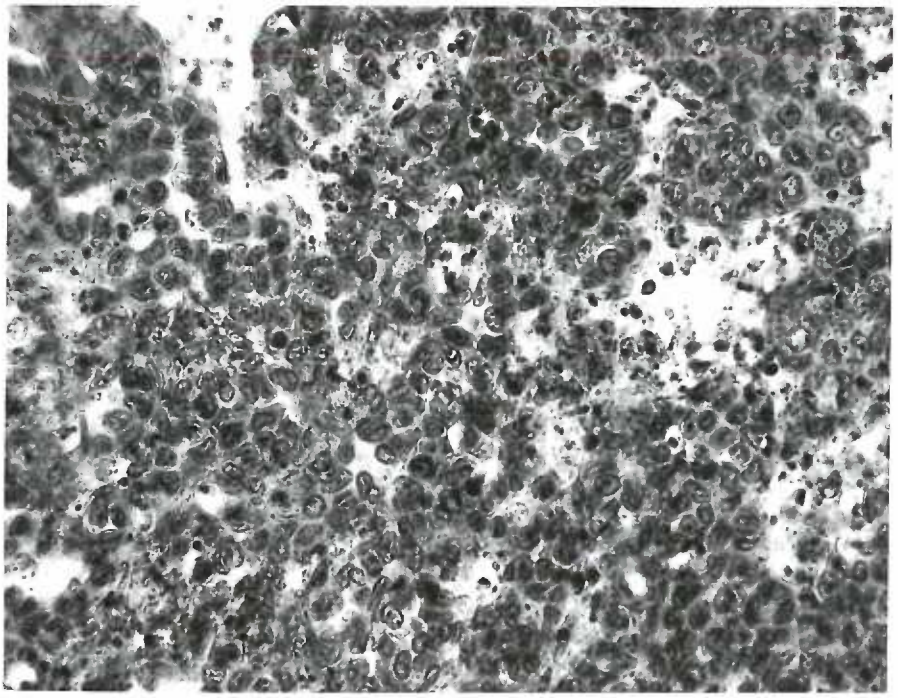
34



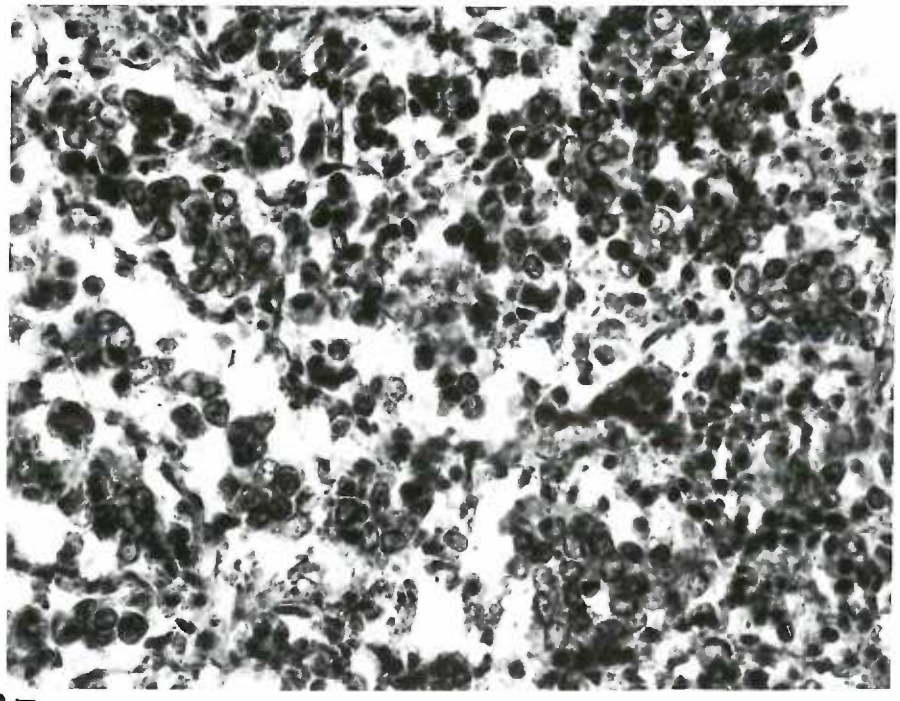
35

Figure 36. Cultured in medium 199 with 100 micrograms per ml. of cortisol and 50 micrograms per ml. of testosterone for three days. No tendency for karyolysis. 330X.

Figure 37. Cultured in medium 199 with 50 micrograms per ml. of cortisol and 50 micrograms per ml. of testosterone for three days. Note tendency for karyolysis. 330X.



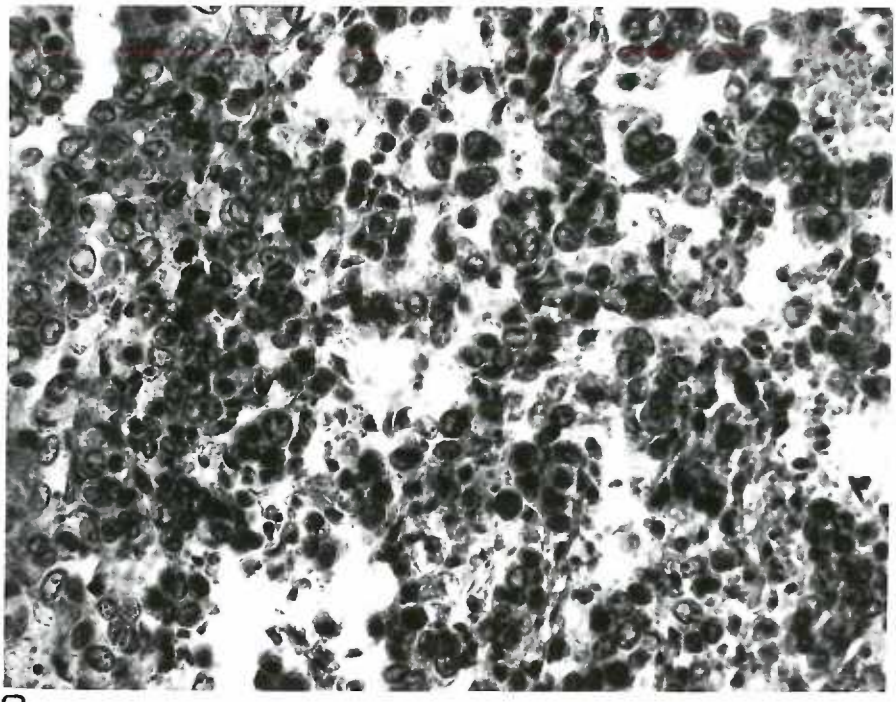
36



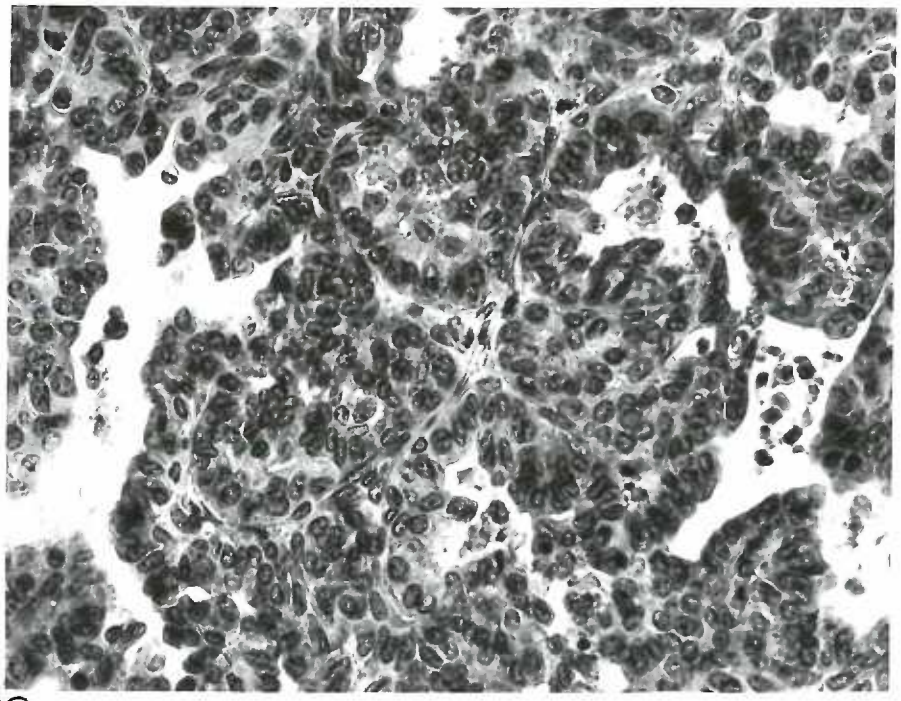
37

Figure 38. Cultured in medium 199 with 10 micrograms per ml. of cortisol and 50 micrograms per ml. of testosterone for three days. 330X.

Figure 39. Cultured in medium 199 with 100 micrograms per ml. of insulin, 25 micrograms per ml. of cortisol, and 1.0 micrograms per ml. of testosterone for three days. 330X.



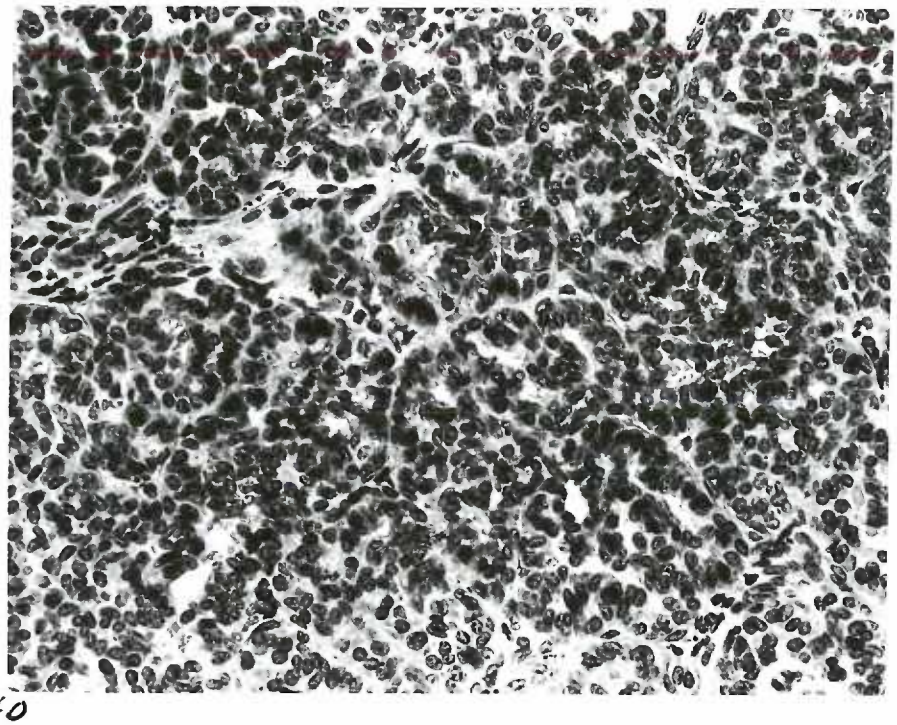
38



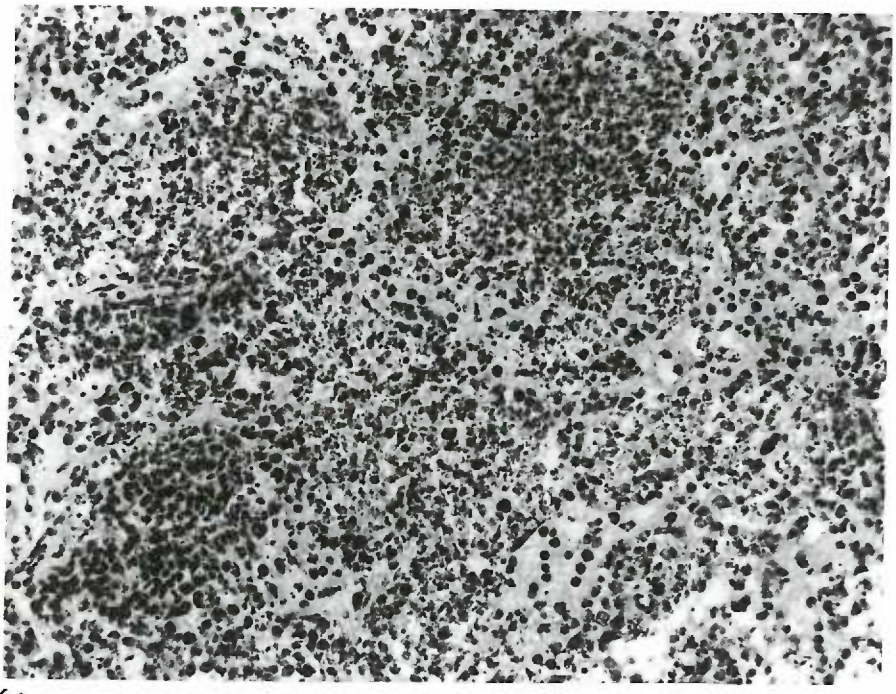
39

Figure 40. Cultured in medium 199 with 100 micrograms per ml. of insulin, 25 micrograms per ml. of cortisol, and 10 micrograms per ml. of testosterone for three days. 275X.

Figure 41. Cultured in medium 199 with 100 micrograms per ml. of insulin, 24 micrograms per ml. of cortisol, and 100 micrograms per ml. of testosterone for three days. 275X.



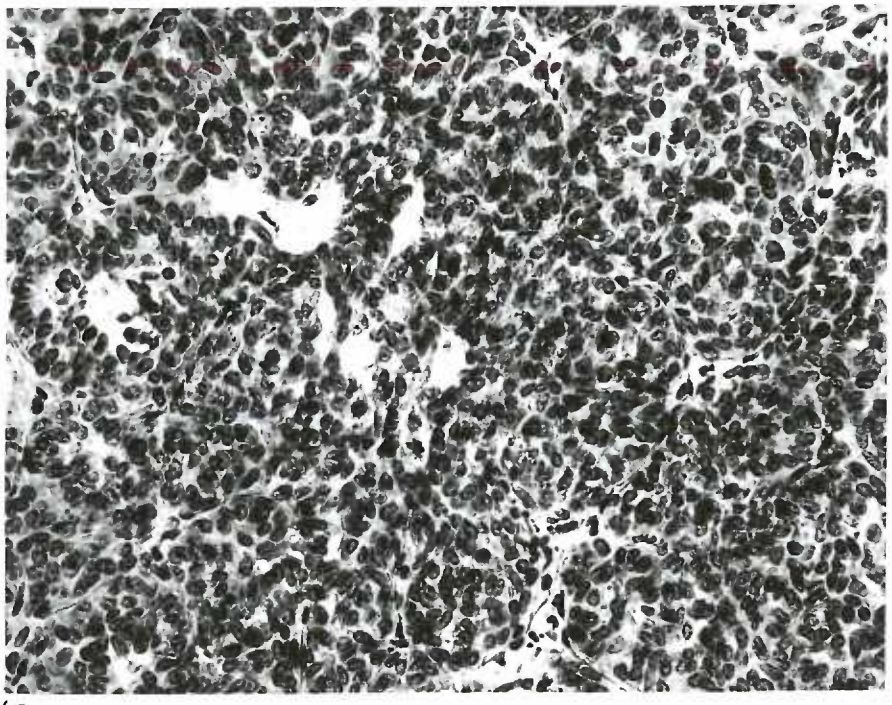
40



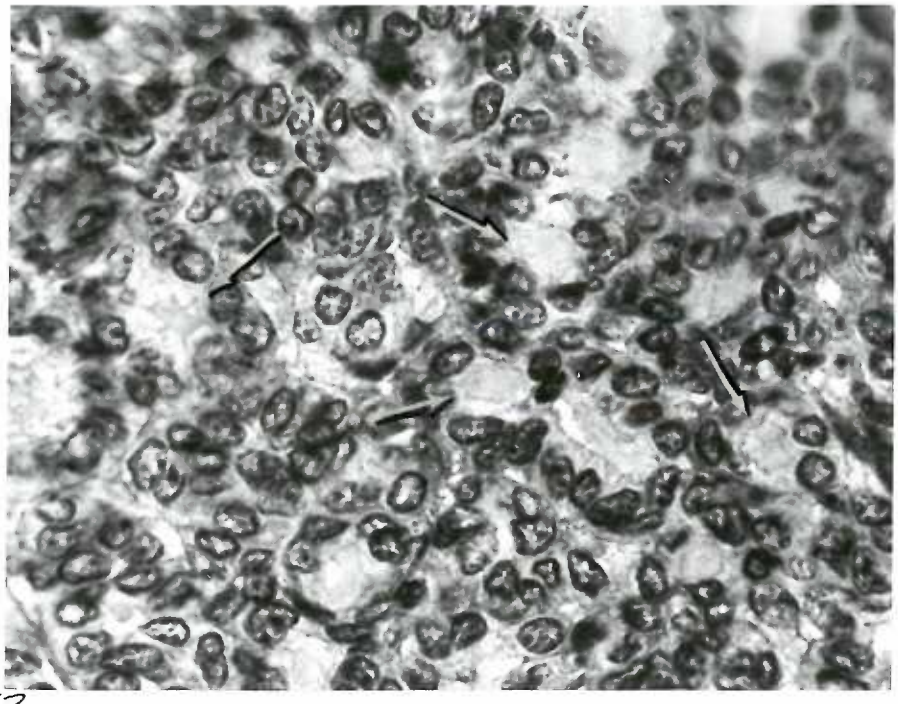
41

Figure 42. Cultured in medium 199 with 100 micrograms per ml. of insulin and 25 micrograms per ml. of cortisol for three days. 375X.

Figure 43. Cultured in medium 199 with 100 micrograms per ml. of insulin, 12 micrograms per ml. of cortisol, and 0.6 micrograms per ml. of estradiol-17B for three days. Note secretions in lumens (arrows). 665X.



42



43

Letter codes for Electron Micrographs

(Figures 44-66)

N - Nucleus

CM - Cell membrane

NM - Nuclear membrane

G - Golgi apparatus

M - Mitochondria

R - Ribonucleoprotein particles

V - Virus-like particles

NC - Nucleolus

DN - Degenerating nucleus

DC - Degenerating cell

L - Lumen

MV - Microvilli

F - Fat droplet

ER - Ergastoplasm

(All electron micrographs are from tissue fixed in osmium tetroxide embedded in Epon, and stained with lead hydroxide).

Figure 44. Mouse mammary adenocarcinoma. Original tissue, not cultured. Cells are radially arranged around acinar lumen (l). Very little membranous ergastoplasm is present. There are many virus-like particles (V). Mitochondria (M) are well-formed and have numerous cristae and dense matrices. 11000X.

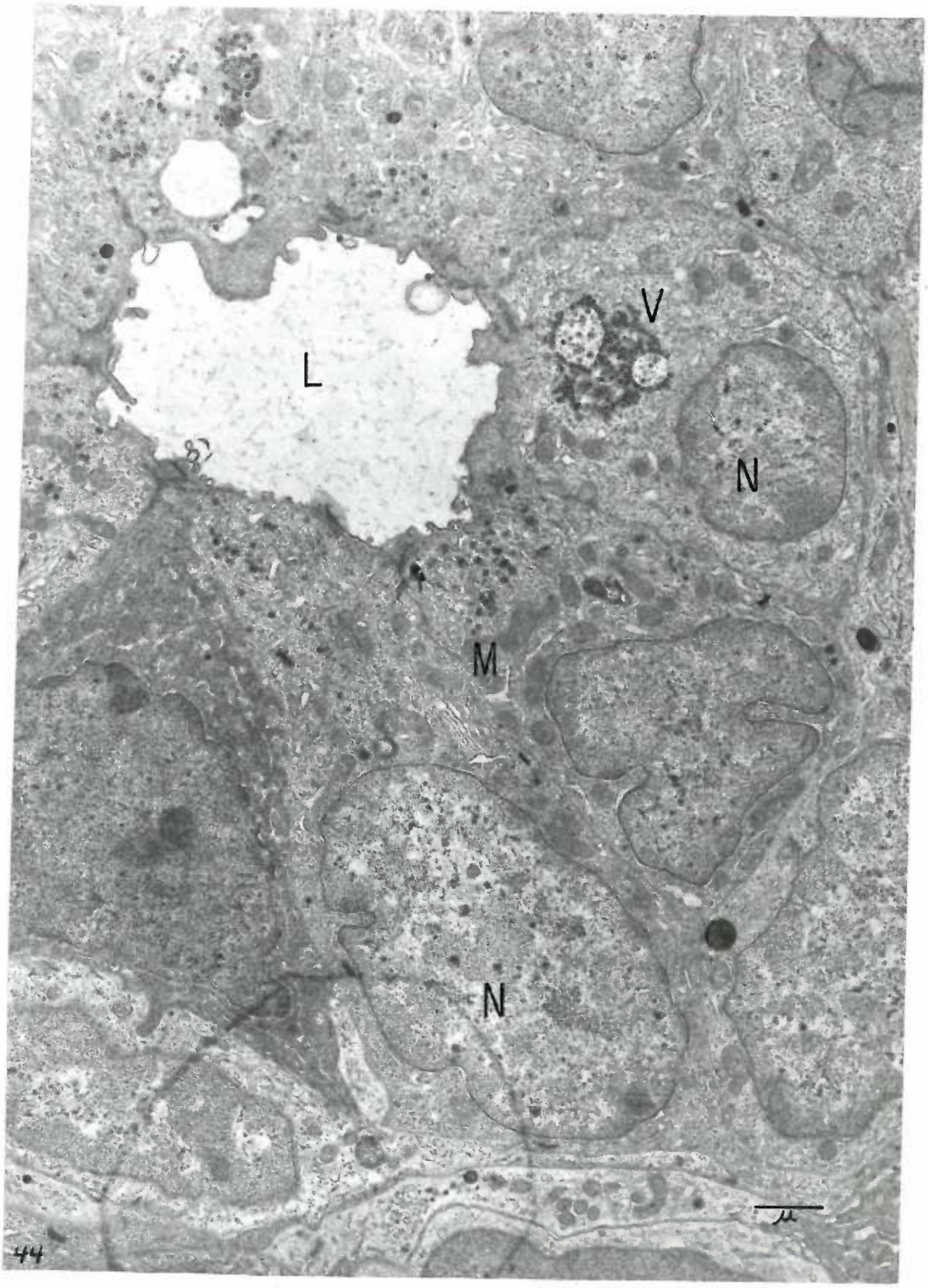

L
V
N
M
N
μ
44

Figure 45. Mouse mammary adenocarcinoma. Original tissue, not cultured. Solid sheet of cells with small lumen (L.) at top containing virus-like particles (V). 11000X.

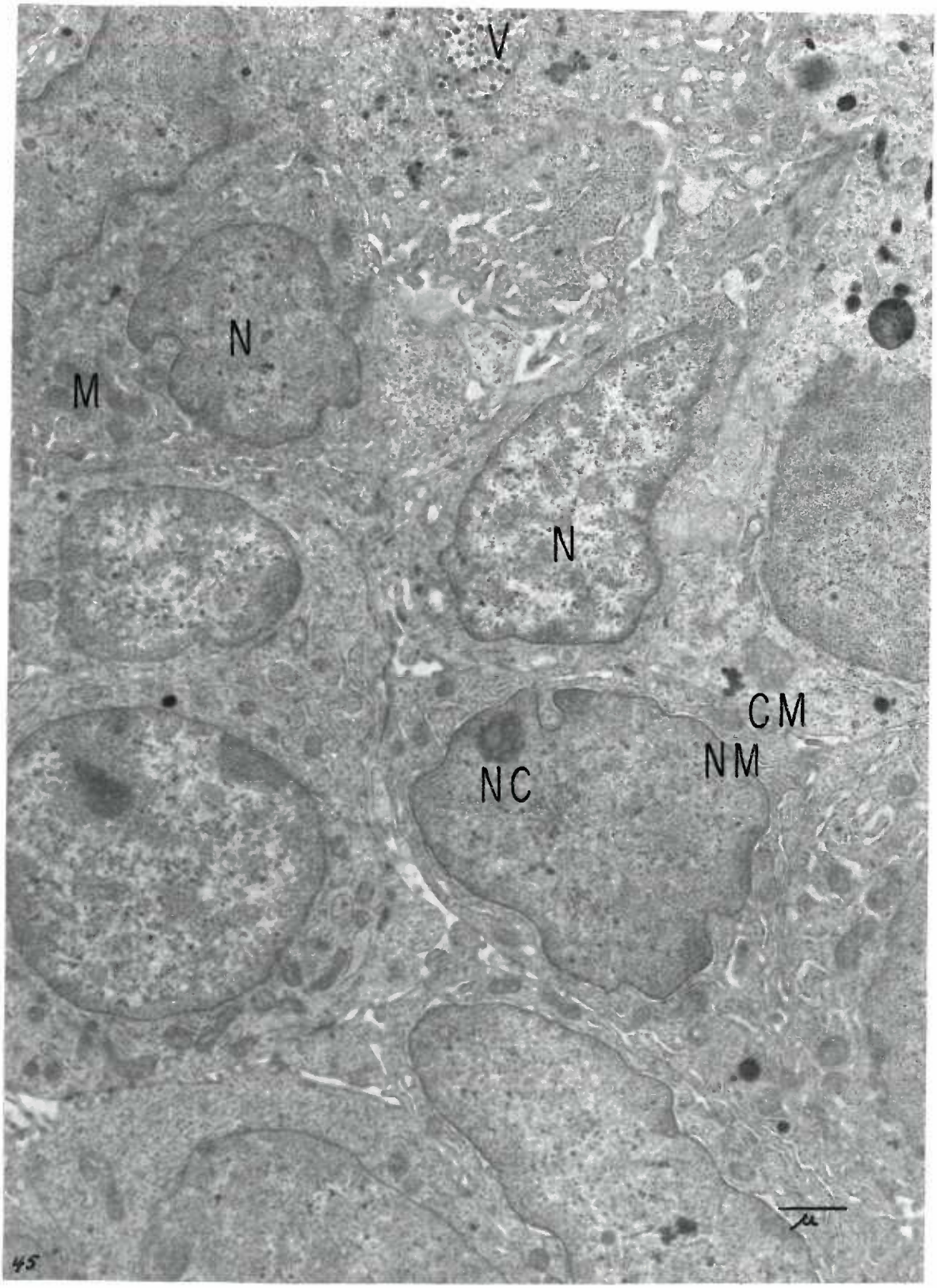

V
N
M
N
CM
NM
NC
μ
45

Figure 46. Mouse mammary adenocarcinoma. Original tissue, not cultured. Numerous virus-like particles of the intracellular type are present. These were frequently located around a vacuole (lower). Cytoplasm has numerous ribonucleoprotein particles (R) and a few ergastoplasmic sacs (ER). 50000X.

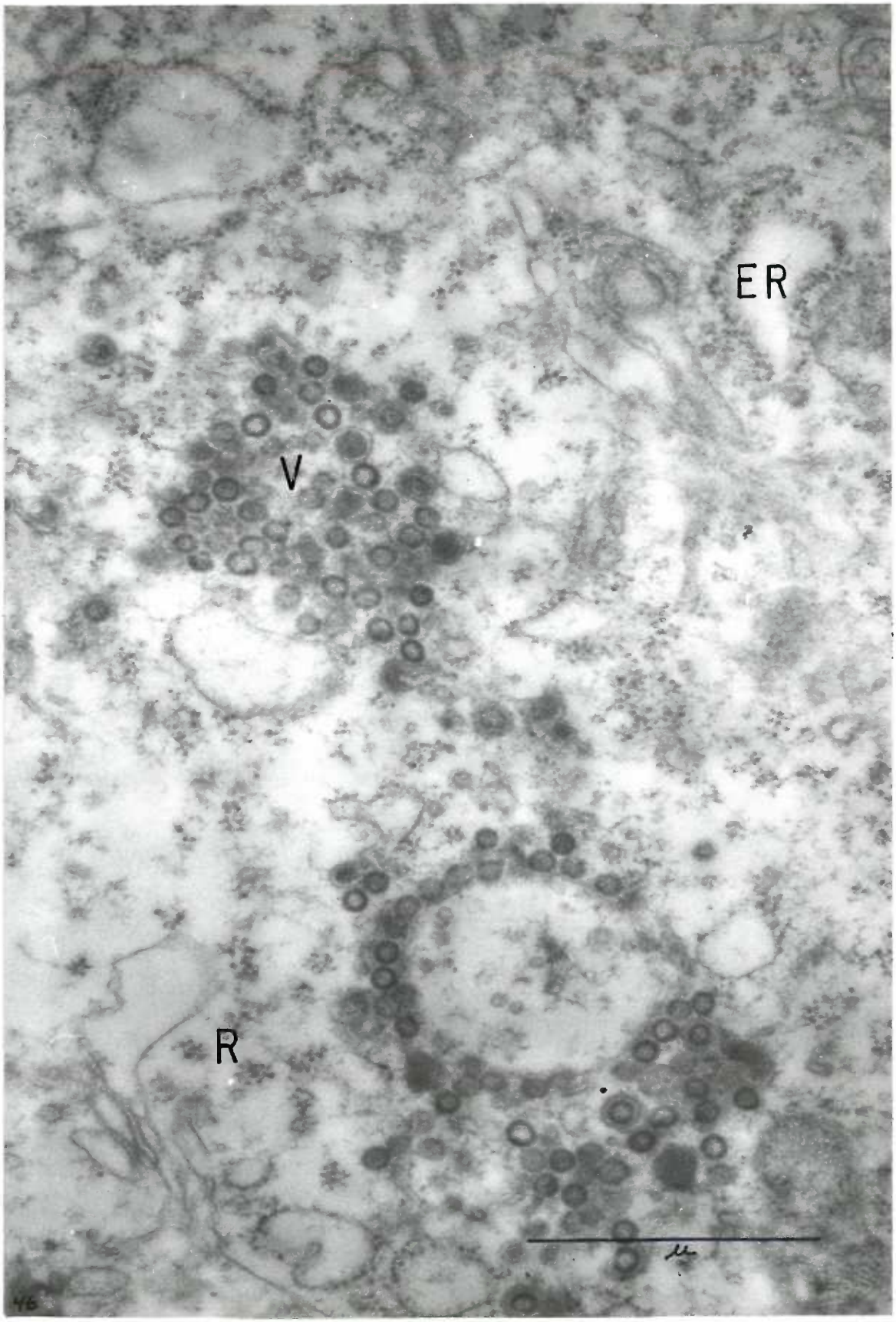

ER
V
R
46

Figure 47. Mouse mammary adenocarcinoma. Control tissue. Cultured in medium 199 alone for one day. Small lumen (L.) centrally. Degenerating cell (DC) at bottom right. 12000X.

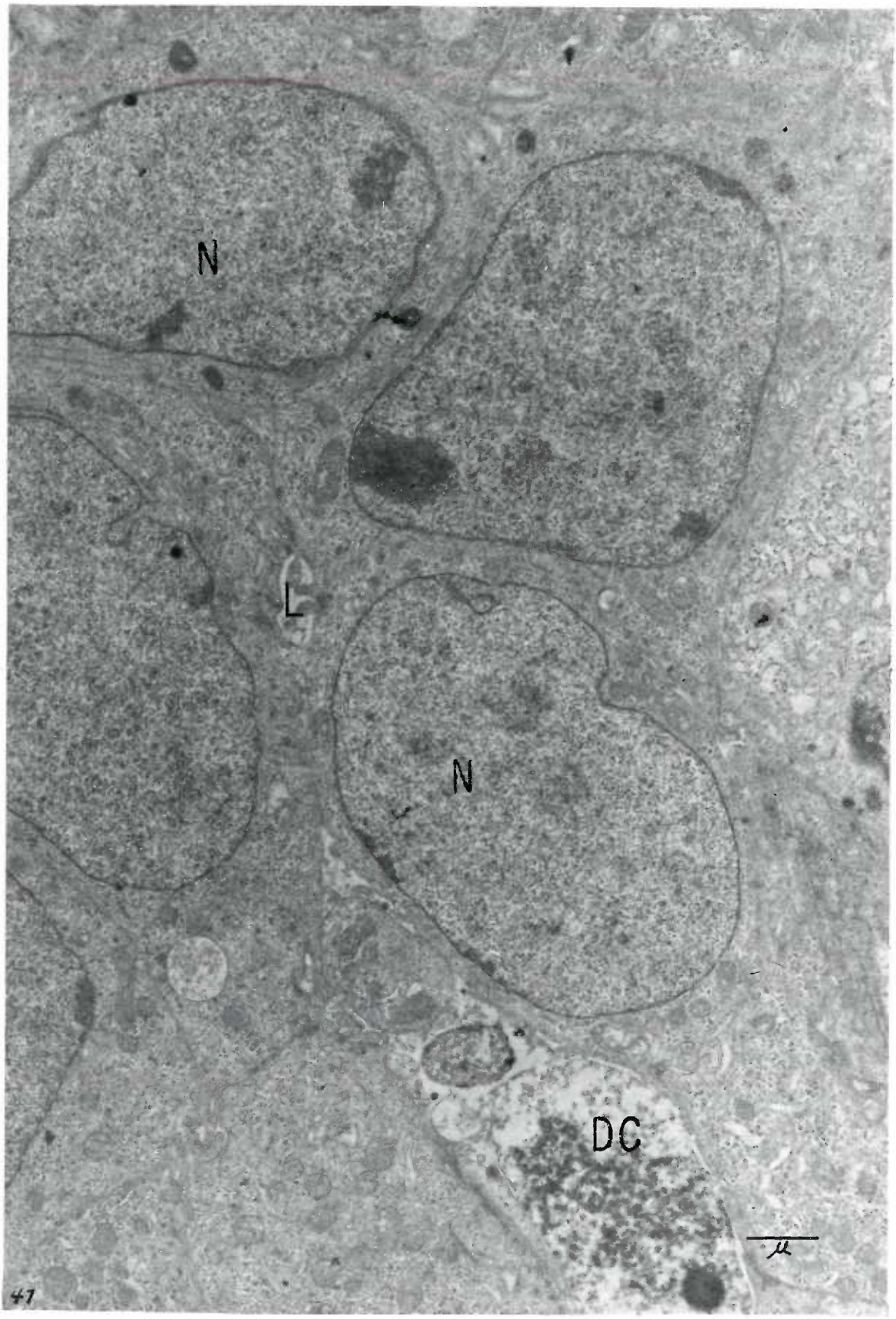

N
L
N
DC
μ
47

Figure 48. Mouse mammary carcinoma. Control tissue. Cultured in medium 199 alone for one day. Various intracellular inclusions are present, two of which are indicated by arrows. Numerous ribosomes (R). 48000X.

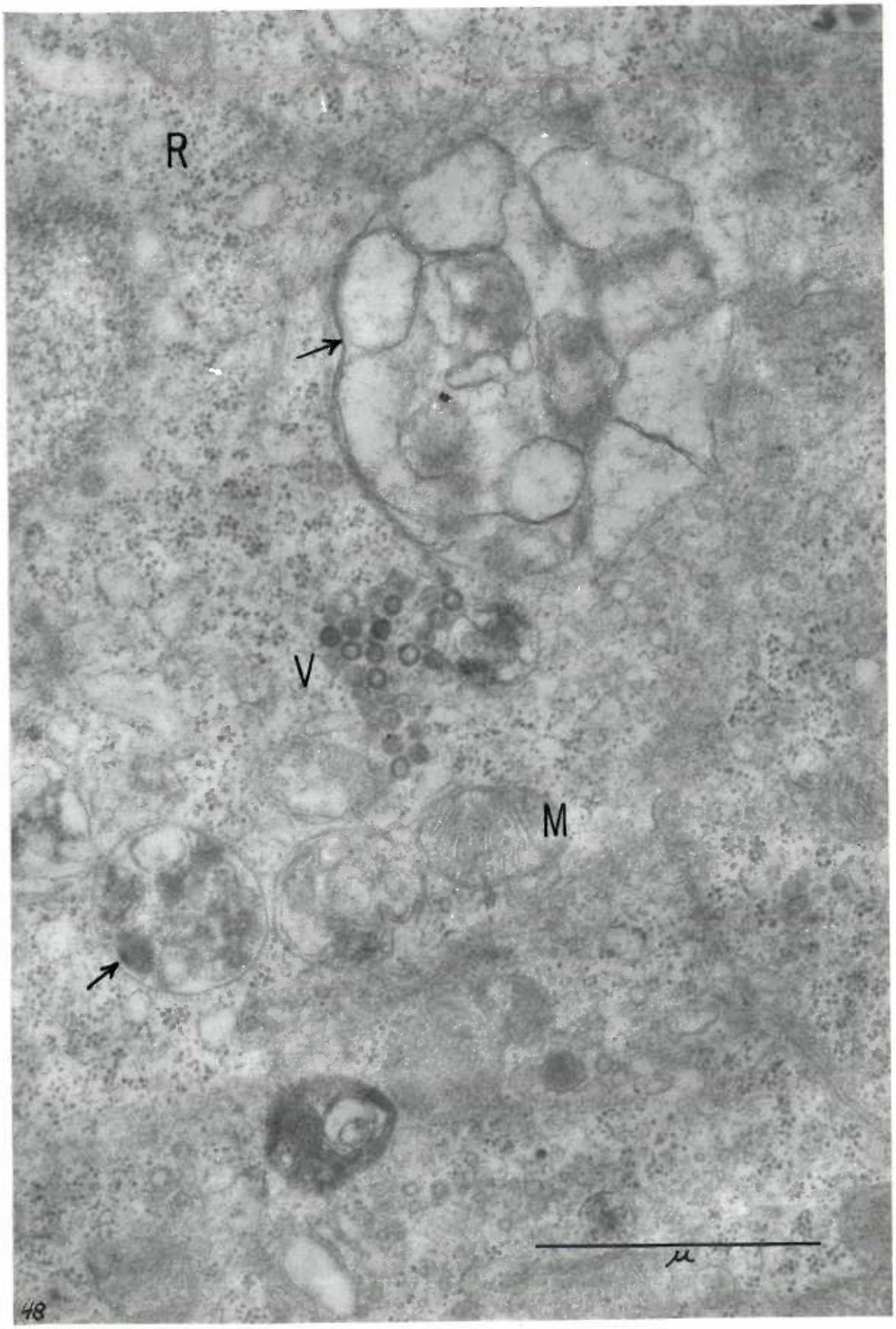
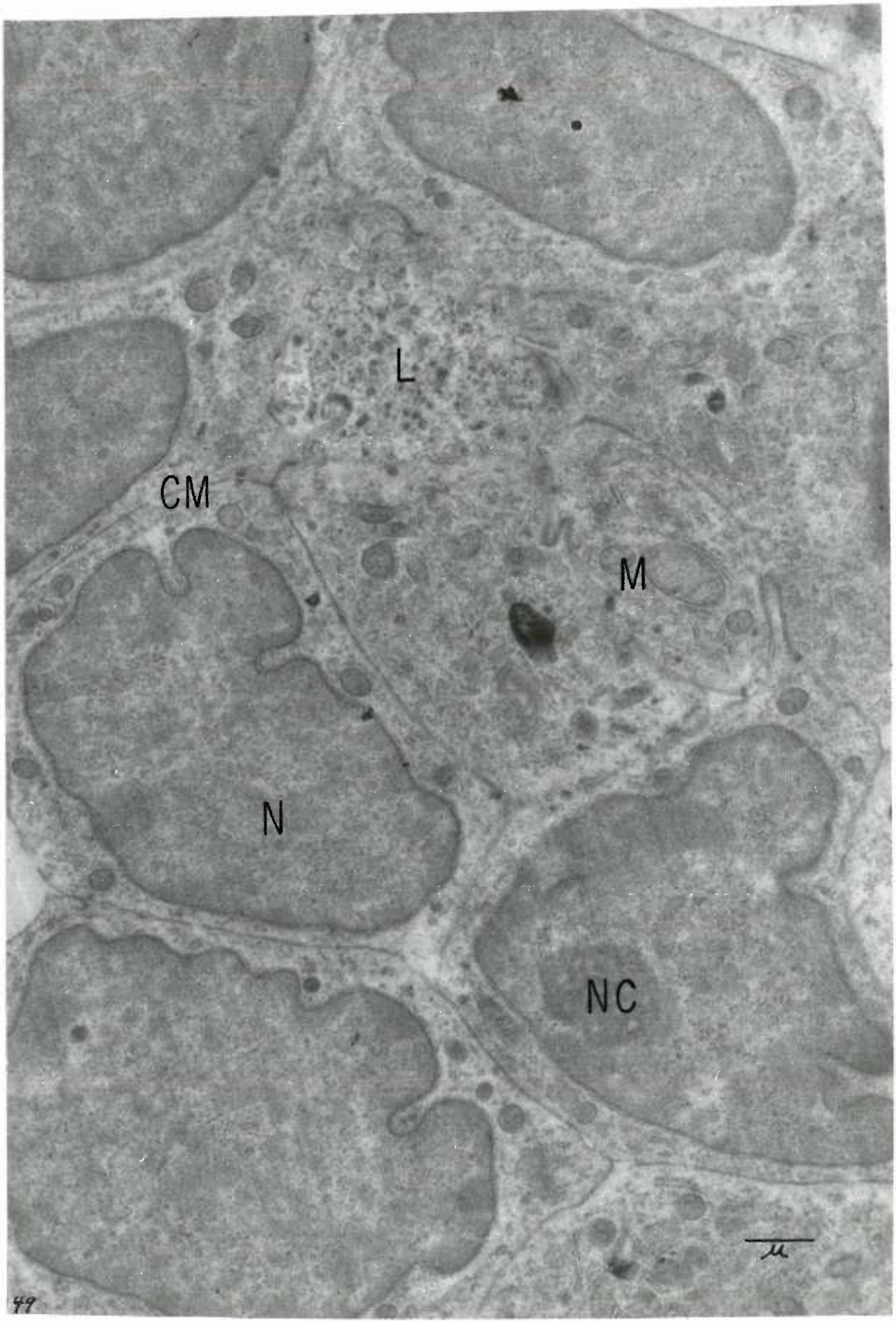

R
V
M
μ
48

Figure 49. Mouse mammary adenocarcinoma. Control tissue. Cultured in medium 199 alone for three days. 12000X.



49

Figure 50. Mouse mammary adenocarcinoma. Control tissue. Cultured in medium 199 alone for three days. 12000X.

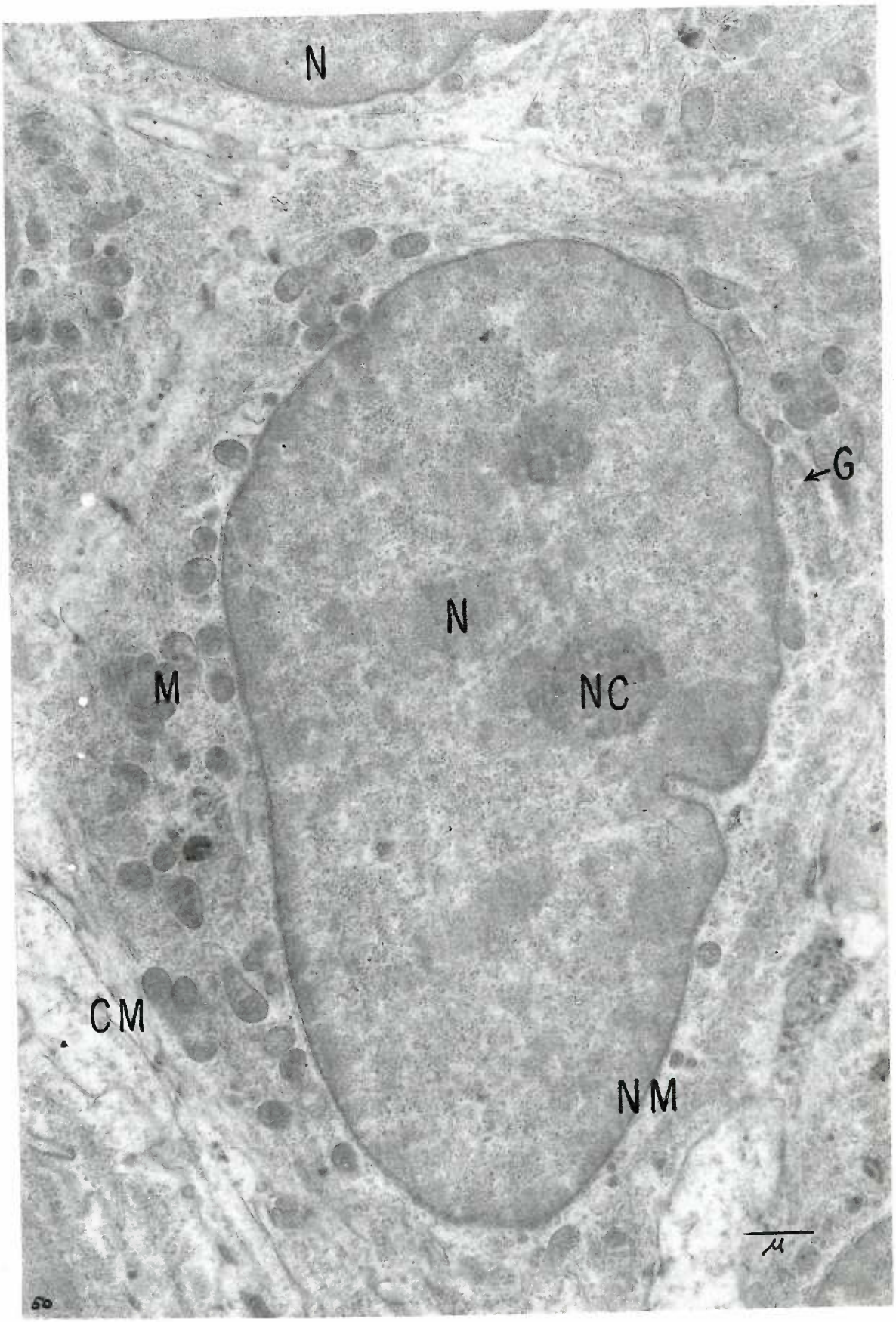

N
G
N
M
NC
CM
NM
μ
50

Figure 51. Mouse mammary adenocarcinoma. Control tissue. Cultured in medium 199 alone for three days. Numerous virus-like particles (V) of the extracellular type are present in the lumen (L) which occupies the upper two-thirds of the micrograph. Cell membrane (CM). 41000X.

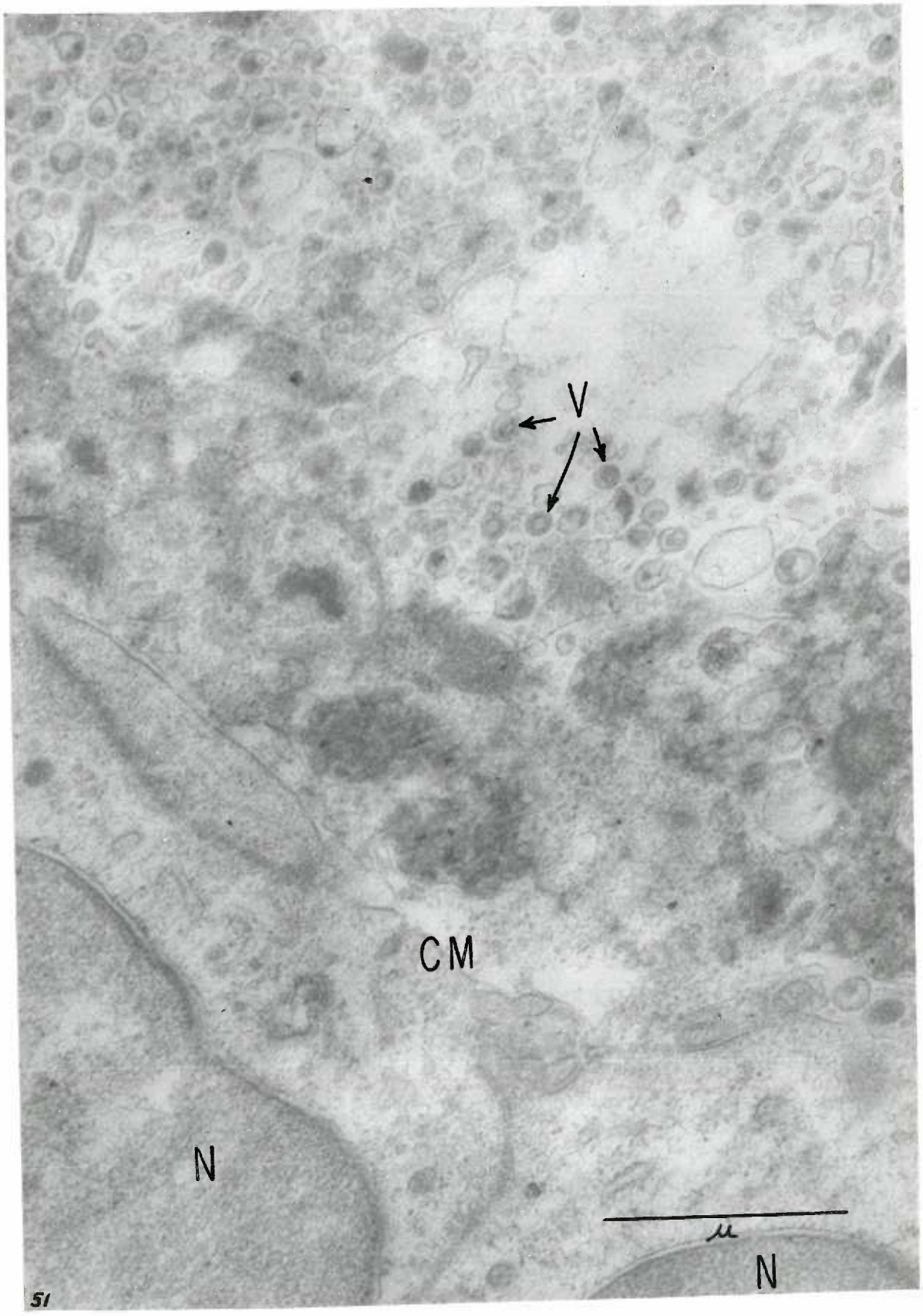

V
CM
N
N
μ
51

Figure 52. Mouse mammary adenocarcinoma. Control tissue. Cultured in medium 199 alone for three days. The dense irregular objects are cytoplasmic bodies of unknown nature. Mitochondria are numerous and of normal morphology. 40000X.

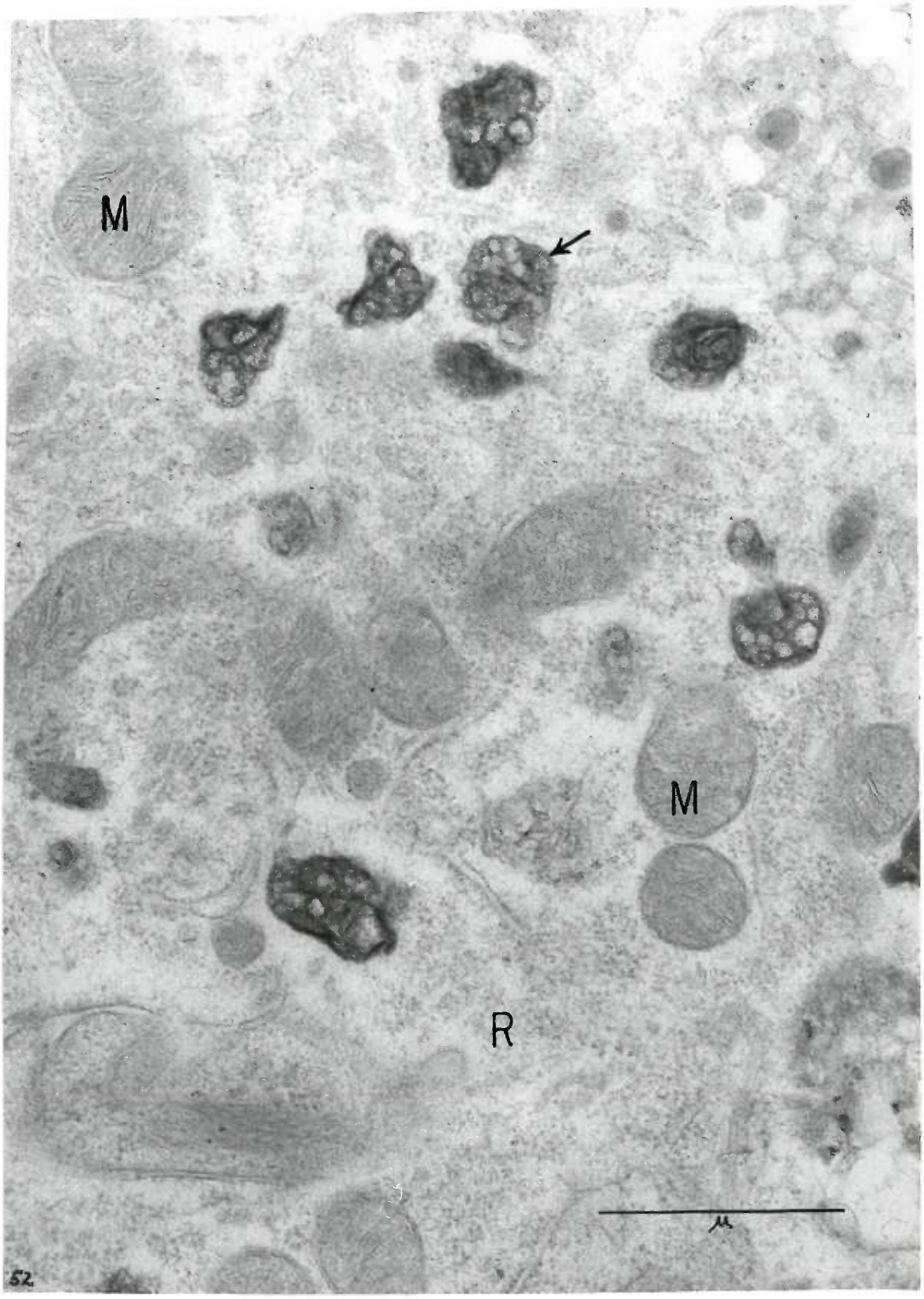

M
M
R
μ
52

Figure 53. Mouse mammary adenocarcinoma. Cultured in medium 199 with 50 micrograms per ml. of testosterone for three days. Note nucleus (N) with irregular coarse densities. Mitochondria (M) are swollen and show decreased numbers of cristae and fragmentation of cristae. Many mitochondria contain ovoid inclusions of high electron density. The large irregular dense objects at the upper left are fragments of karyorrhectic nuclei. The remainder of these necrotic cells consists of unrecognizable granular and membranous debris. 12000X.

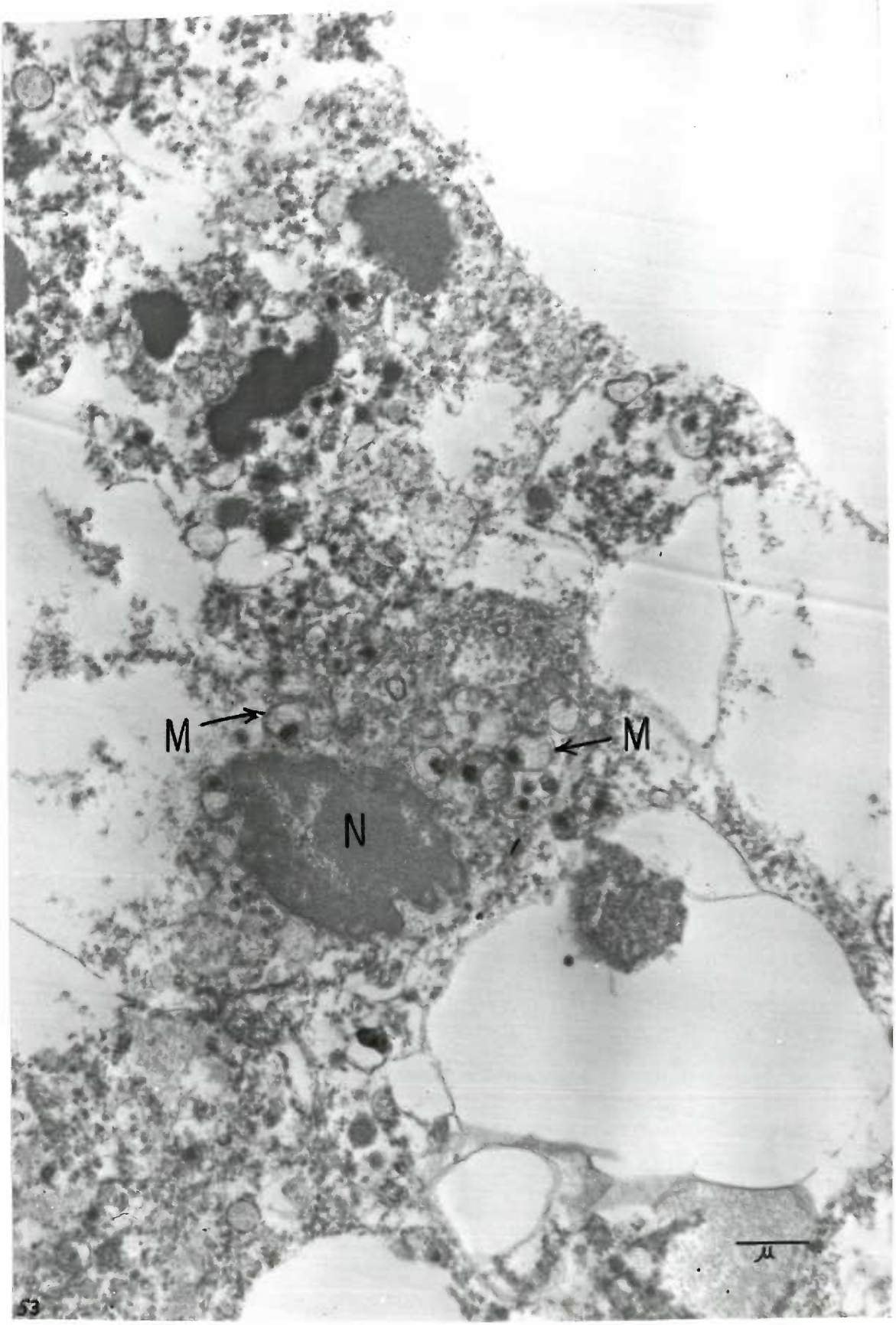
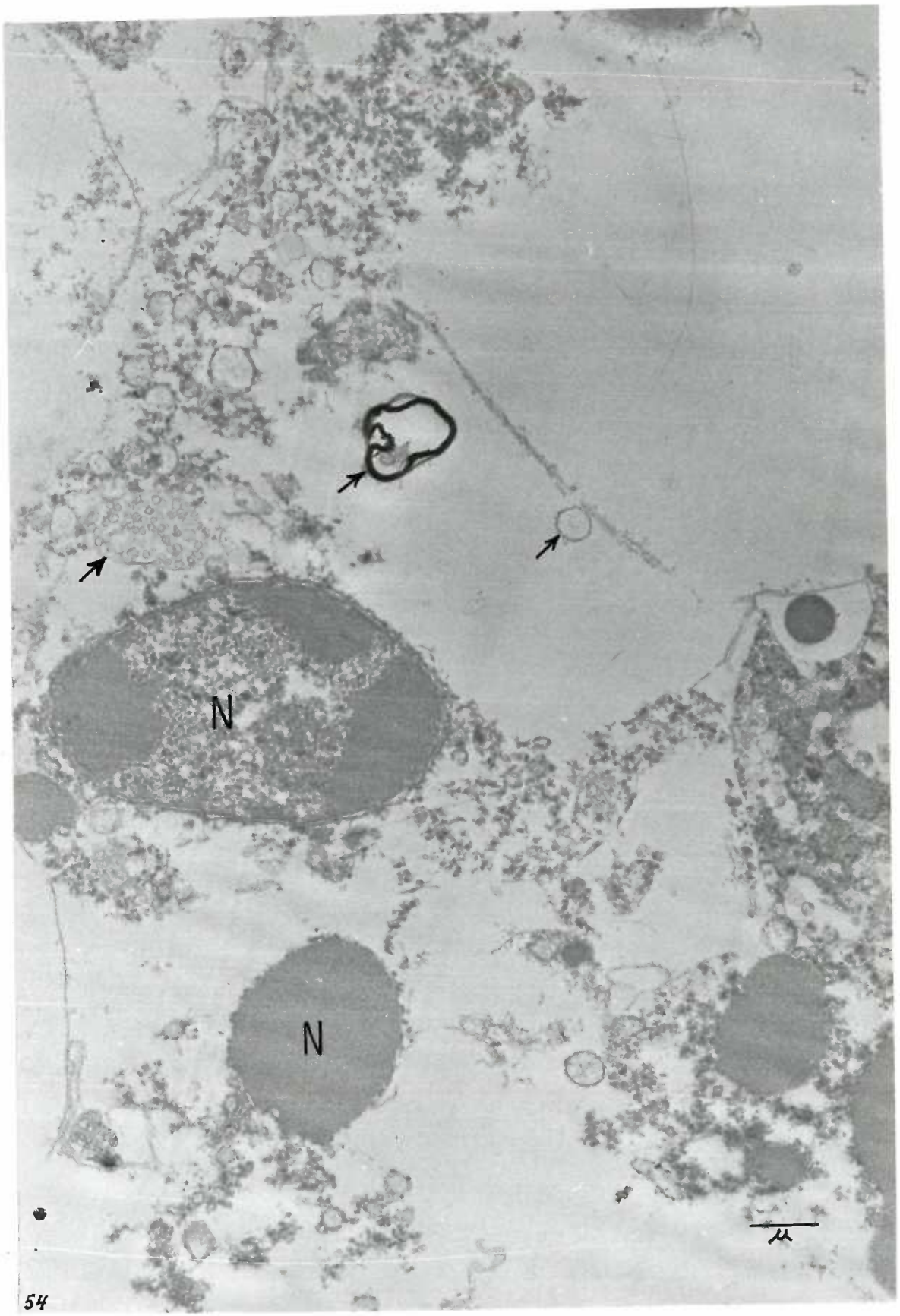

M
M
N
μ
53

Figure 54. Mouse mammary adenocarcinoma. Cultured in medium 199 with 50 micrograms of testosterone for three days. Complete cytolysis. Nuclei (N). Various peculiar objects and vacuolated structures (arrows). 12000X.



54

Figure 55. Mouse mammnary adenocarcinoma. Cultured in medium 199 with 100 micrograms per ml. of insulin and 50 micrograms per ml. of testosterone for three days. Several degenerating cells (upper, middle, and lower) possess granular cytoplasm and dense nuclei (DN). Remainder of cells appear relatively unaffected. 9000X.

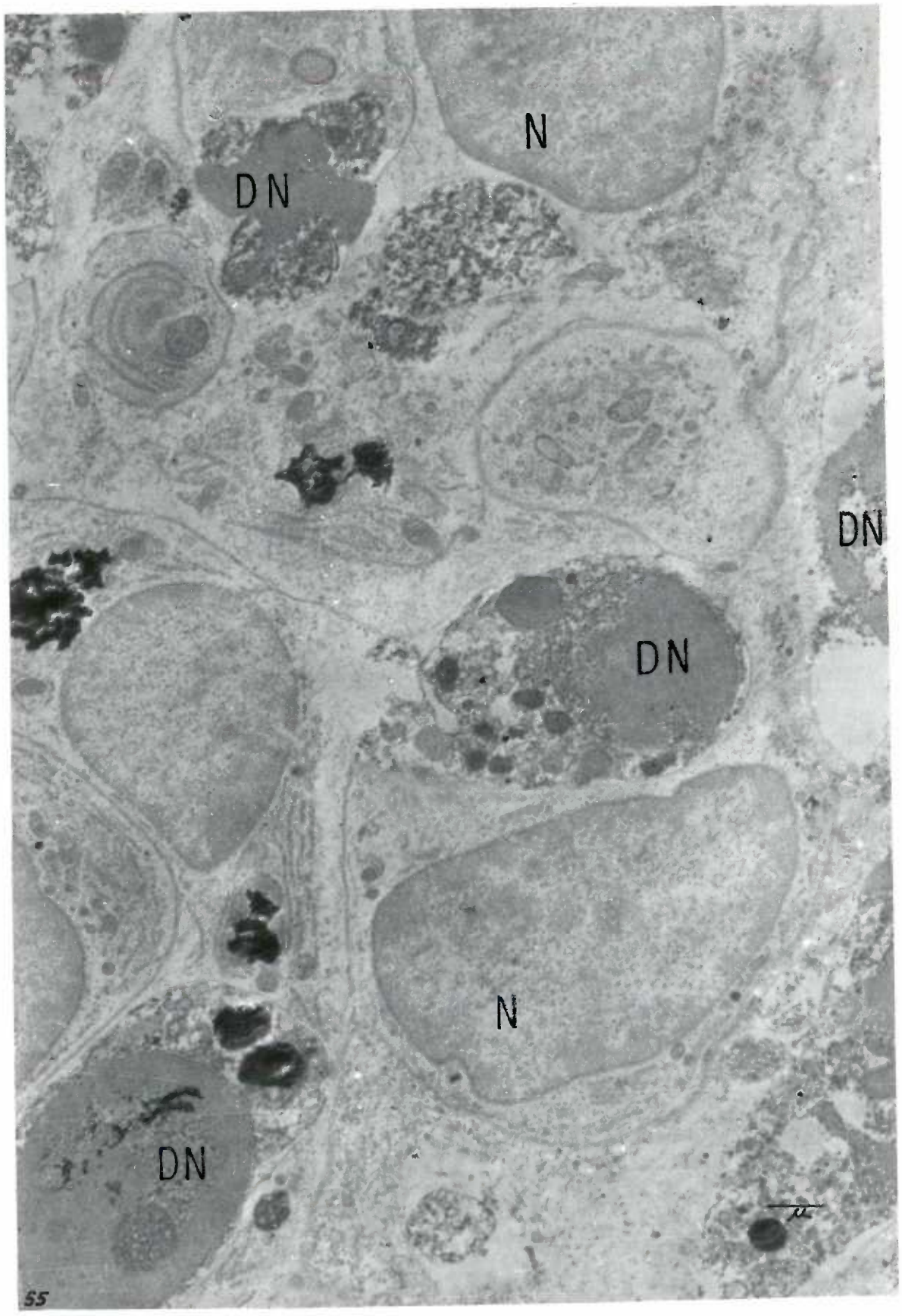

N
DN
DN
DN
N
DN
55

Figure 56. Mouse mammary adenocarcinoma. Cultured in medium 199 with 100 micrograms per ml. insulin, and 50 micrograms per ml. testosterone for three days. Degenerating cell (DC) and nucleus (DN). Remaining cells appear relatively unaffected. 9000X.

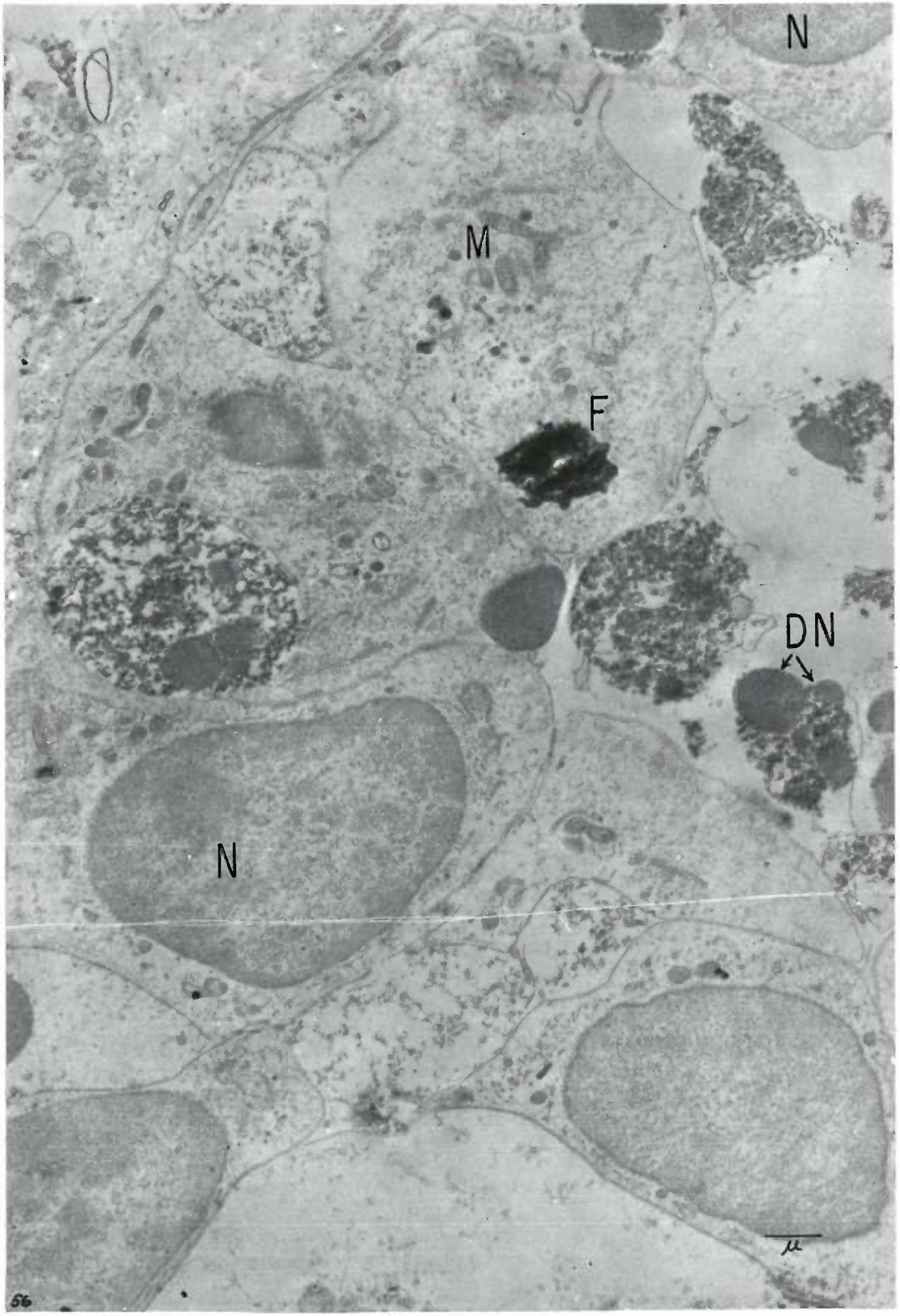

N
M
F
DN
N
μ
56

Figure 57. Mouse mammary adenocarcinoma. Cultured in medium 199 with 100 micrograms per ml. insulin and 50 micrograms per ml. testosterone for 3 days. Portion of surviving cell with fat droplet (F) and numerous ergastoplasmic sacs (ER). Many ribonucleoprotein particles are present. 37000X.

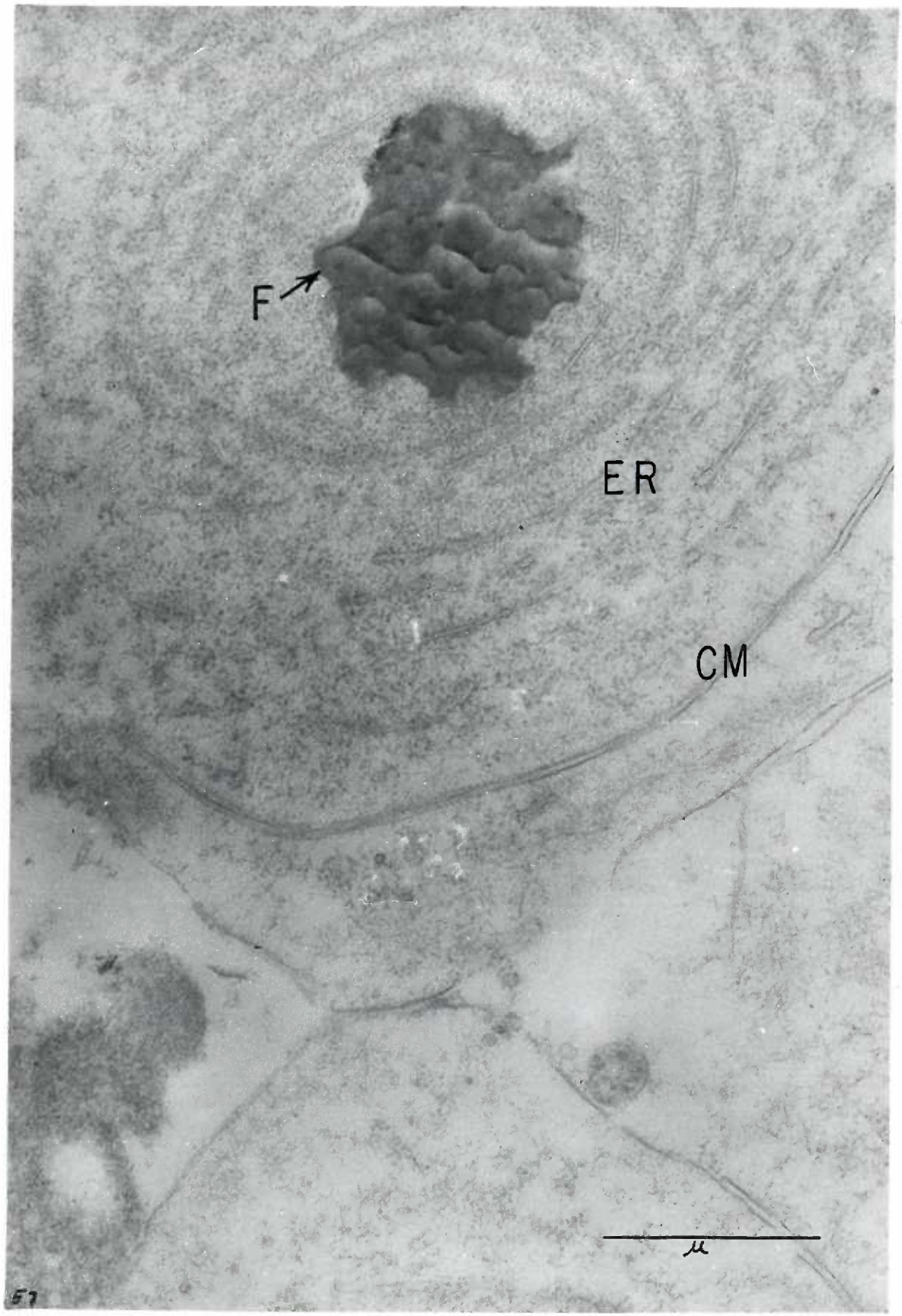

F
ER
CM
μ

Figure 58. Mouse mammary adenocarcinoma. Cultured in medium 199 with 0.1 micrograms per ml. testosterone for one day. Morphology identical with original, uncultured tissue, and with controls in 199 alone. 12500X.

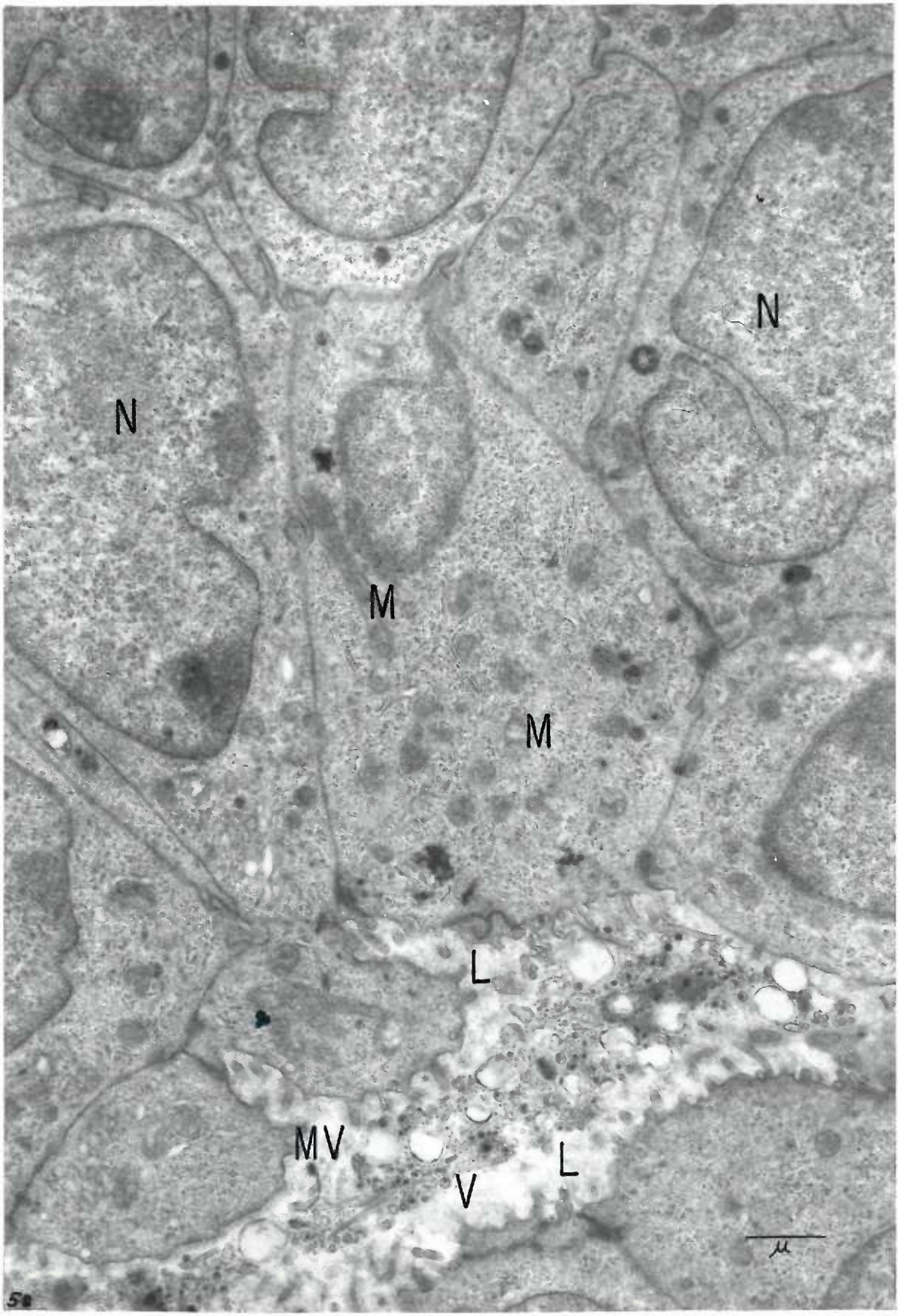
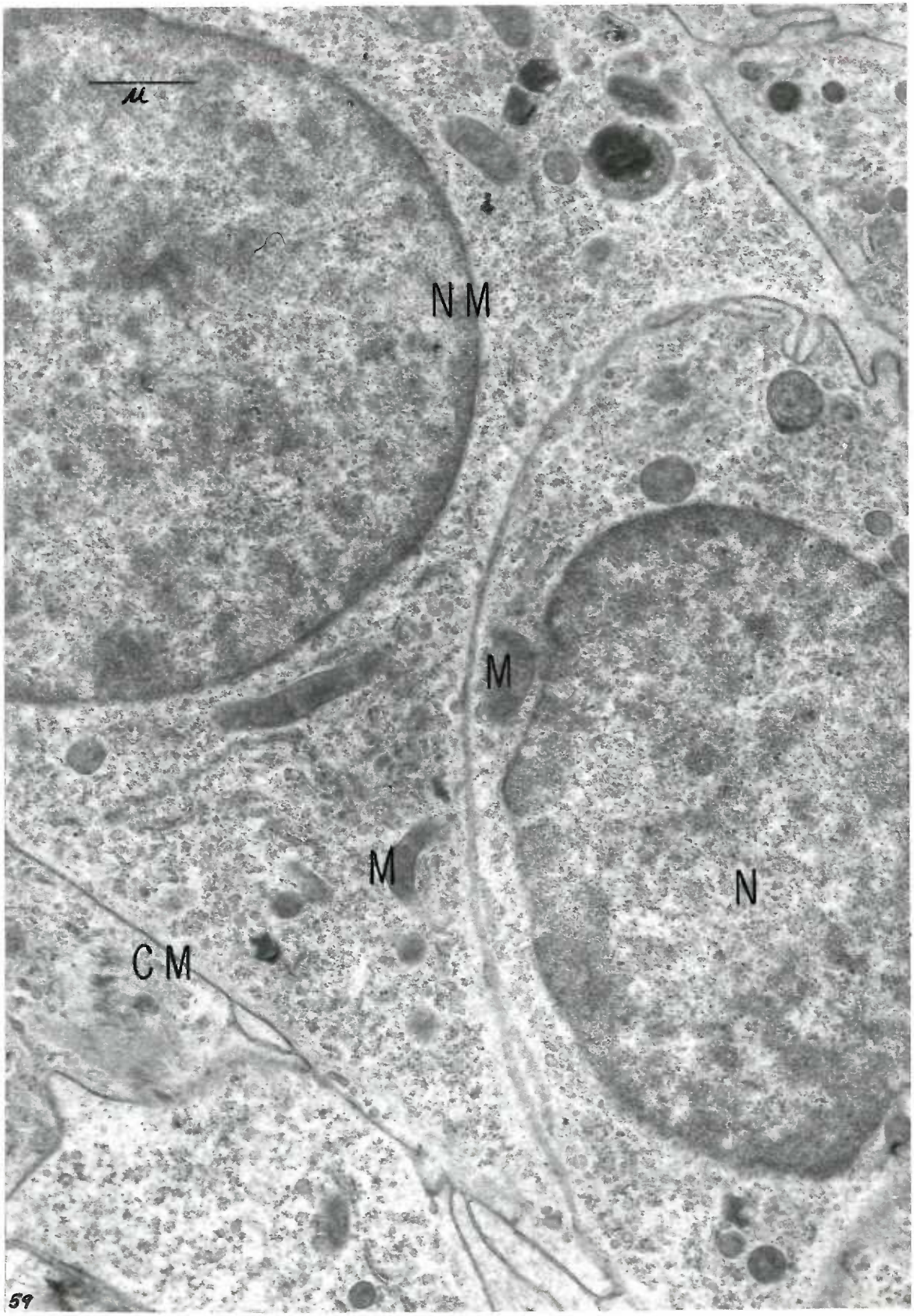

N
N
M
M
L
MV
V
L
μ
5a

Figure 59. Mouse mammary adenocarcinoma. Cultured in medium 199 with 0.1 micrograms per ml. of testosterone for one day. 17000X.



59

Figure 60. Mouse mammary adenocarcinoma. Cultured in medium 199 with 0.1 micrograms per ml. of testosterone for one day. Numerous virus-like particles (arrows) in acinar lumen. Note microvilli at cell surface. 50000X.

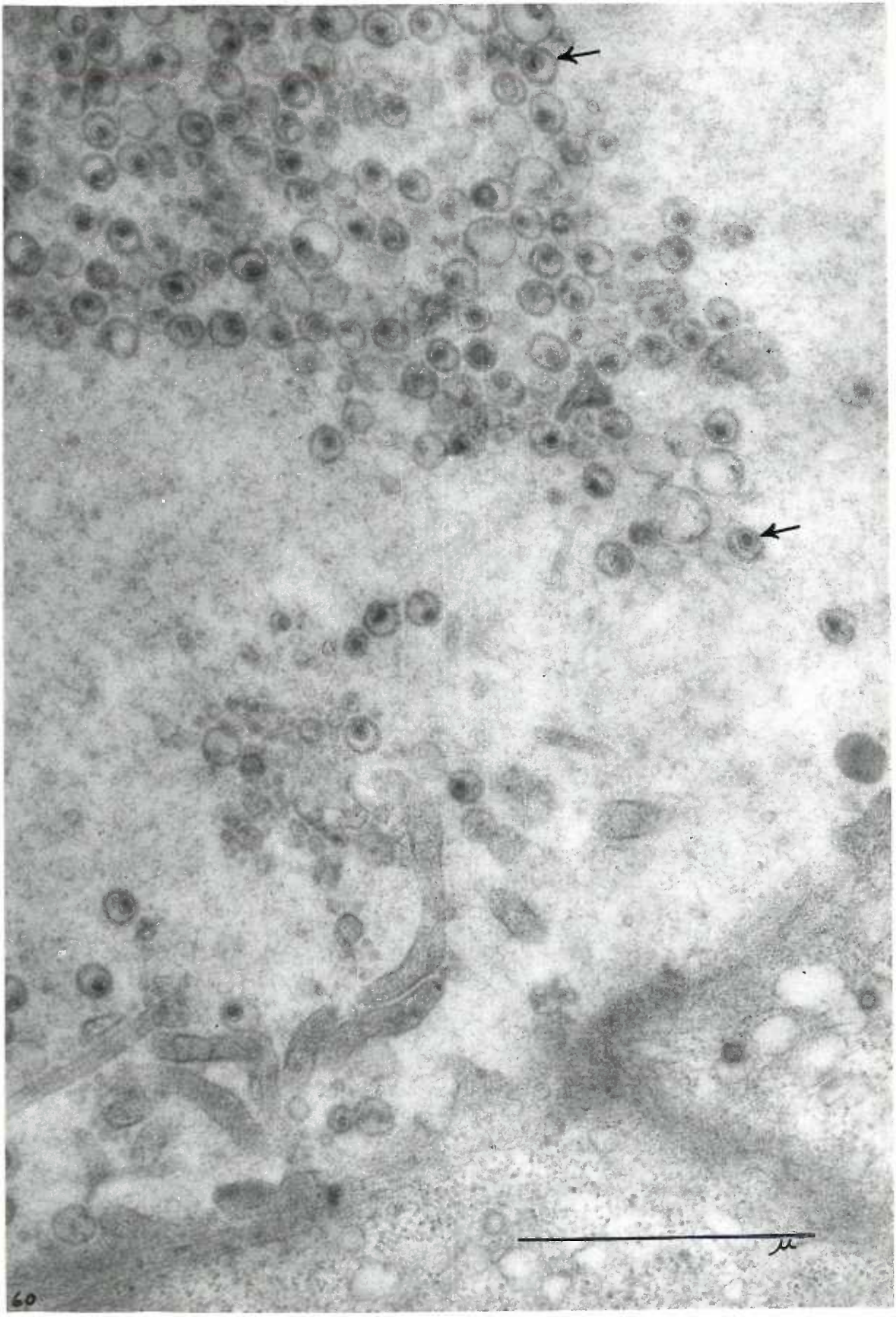
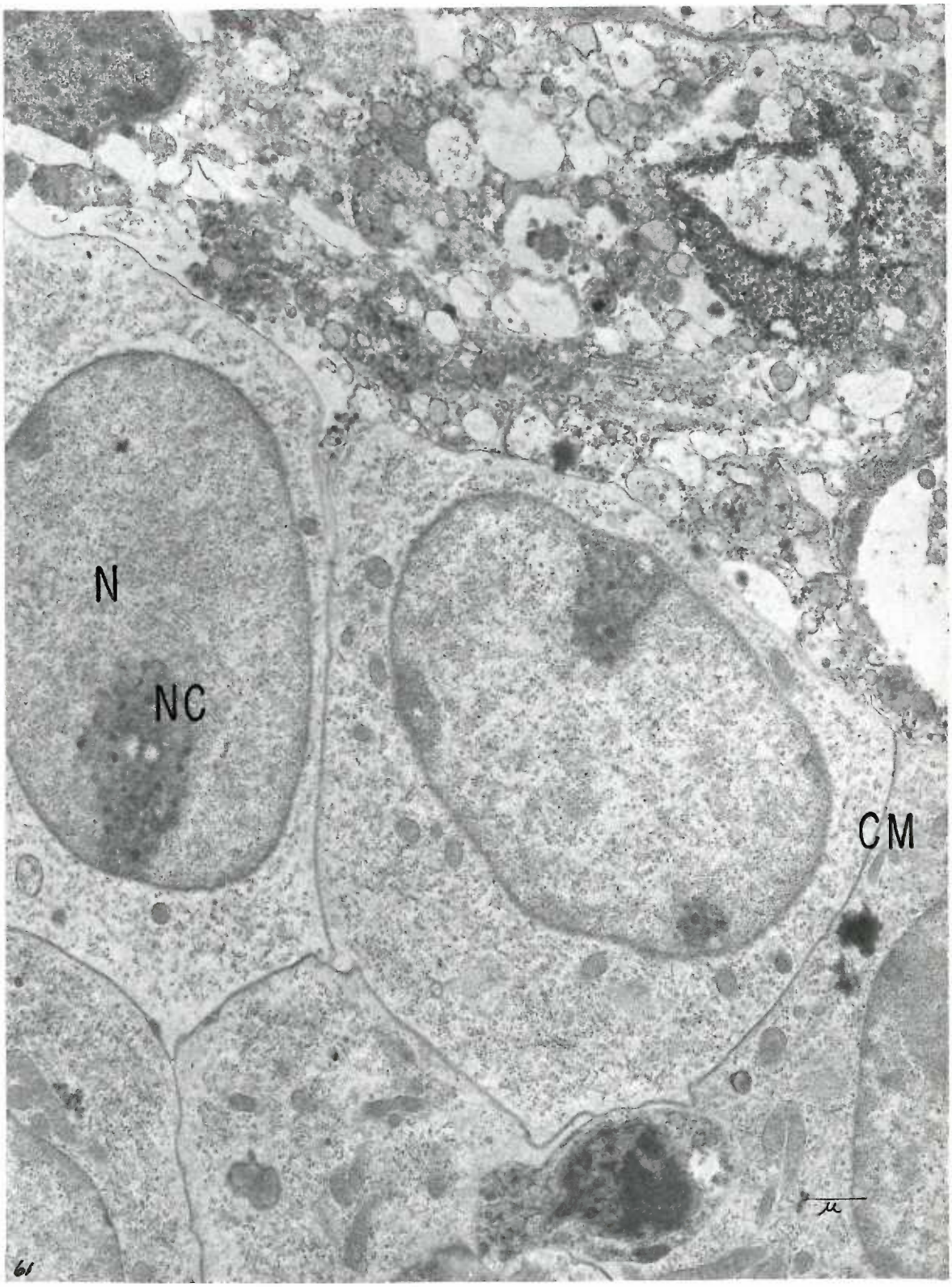

60

Figure 61. Mouse mammary adenocarcinoma. Cultured in medium 199 with 10 micrograms per ml. of testosterone for one day. Cells in lower two-thirds of micrograph are indistinguishable from controls. Necrotic cells in upper one-third. 10000X.



61

Figure 62. Mouse mammary adenocarcinoma. Cultured in medium 199 with 10 micrograms per ml. of testosterone for one day. Numerous virus-like particles of extracellular type in lumen. These particles have a dense central nucleoid and up to three concentrically arranged surrounding membranes (arrow). 62000X.

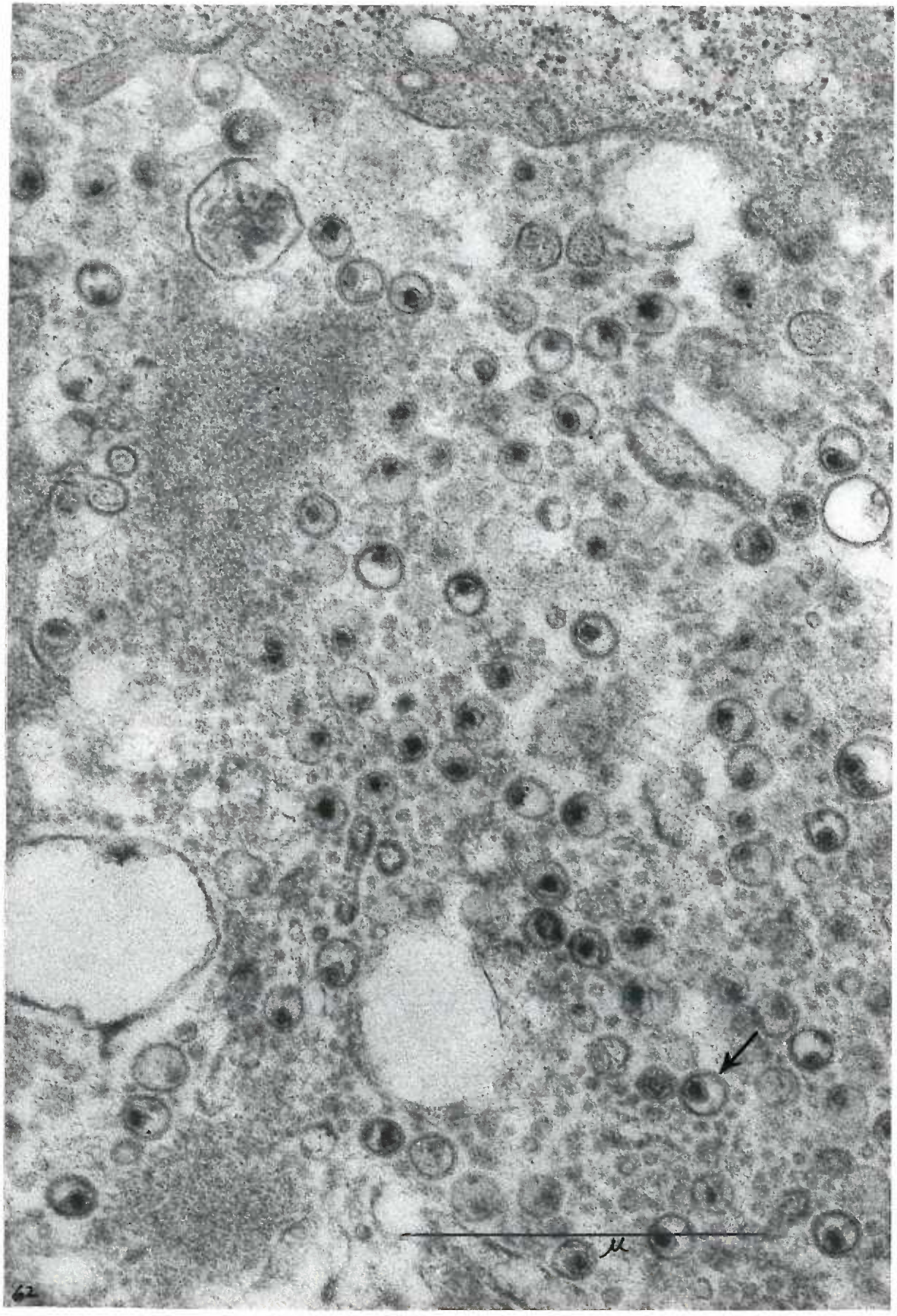

μ
62

Figure 63. Mouse mammary adenocarcinoma. Cultured in medium 199 with 100 micrograms per ml. of testosterone for one day. Note intact cell with fat droplet (F), next to remnants of degenerating and cytolyzed cells. Degenerating nucleus (DN) shows coarse chromatin clumps. 20600X.

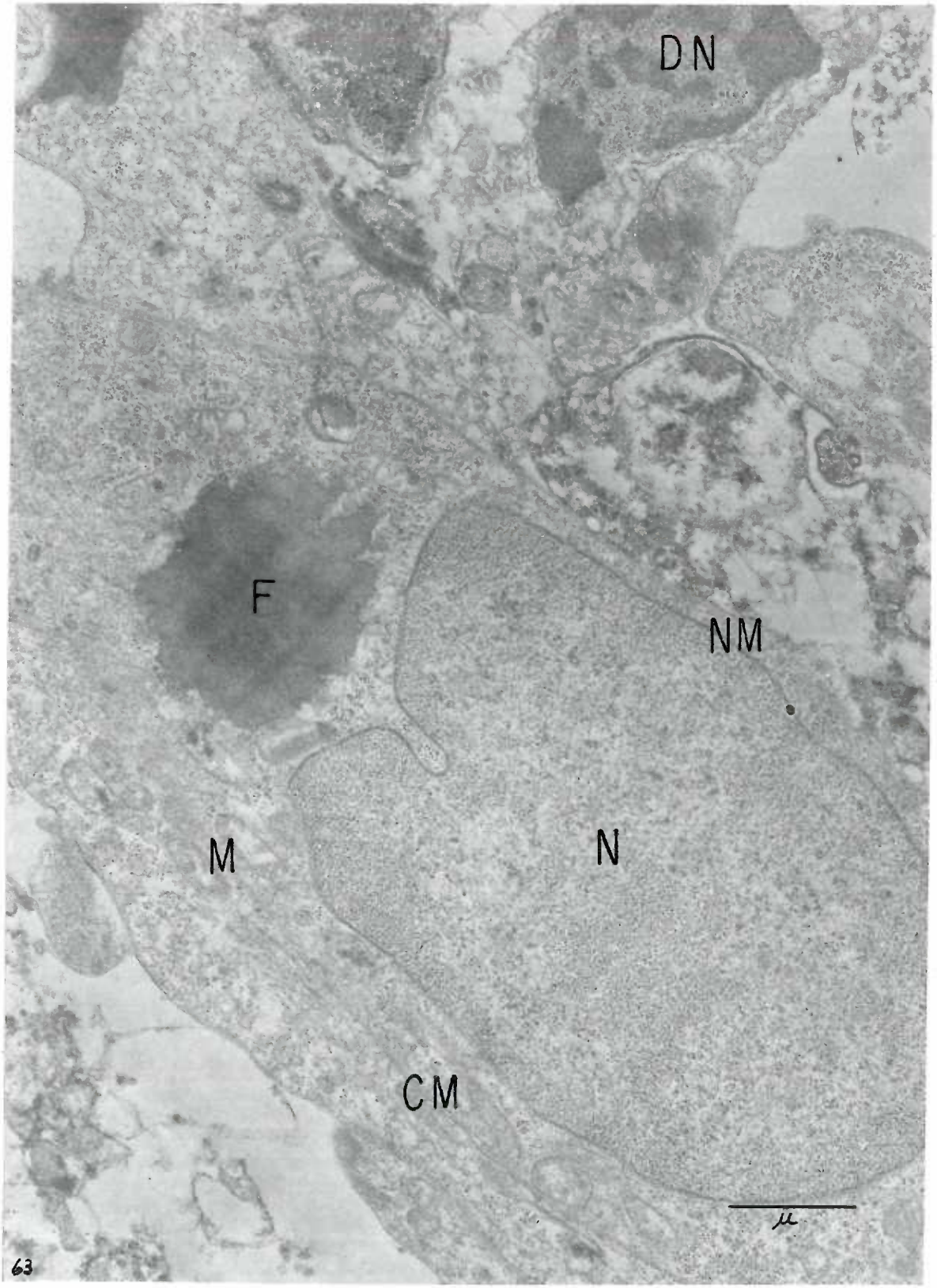

DN
F
NM
M
N
CM
μ
63

Figure 64. Mouse mammary adenocarcinoma. Cultured in medium 199 with 300 micrograms per ml. of testosterone for one day. Groups of several intact cells adjacent to degenerating and cytolyzed cells (DC). Note swollen mitochondrion (M), and vacuolated cell (right middle, DC). 10000X.

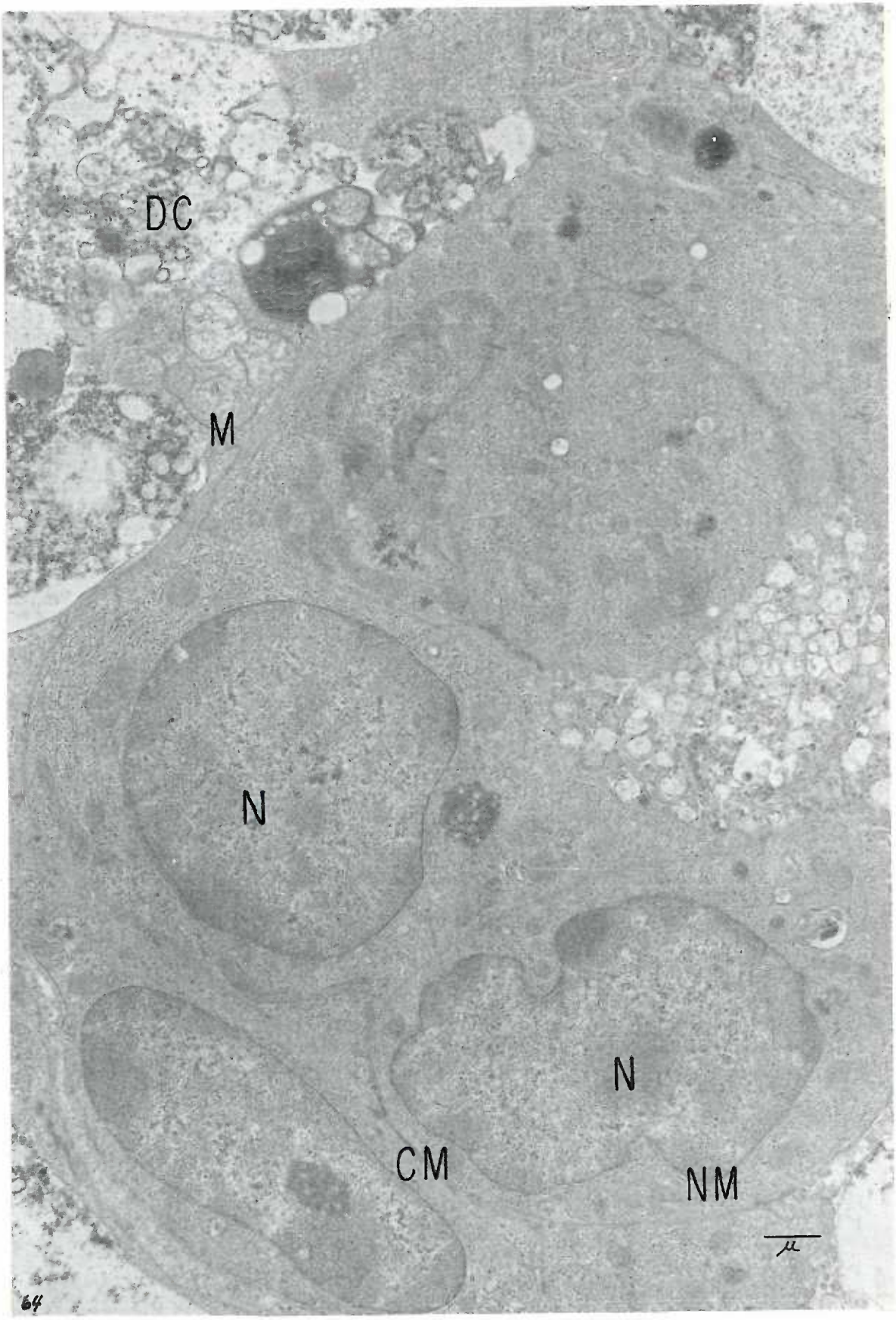

DC
M
N
N
CM
NM
μ
64

Figure 65. Mouse mammary adenocarcinoma. Cultured in medium 199 with 300 micrograms per ml. of testosterone for one day. Nucleus (N) at lower right is fragmenting (karyoffhexis). Nucleus (N) at middle left shows earlier stage of karyorrhexis. 11000X.

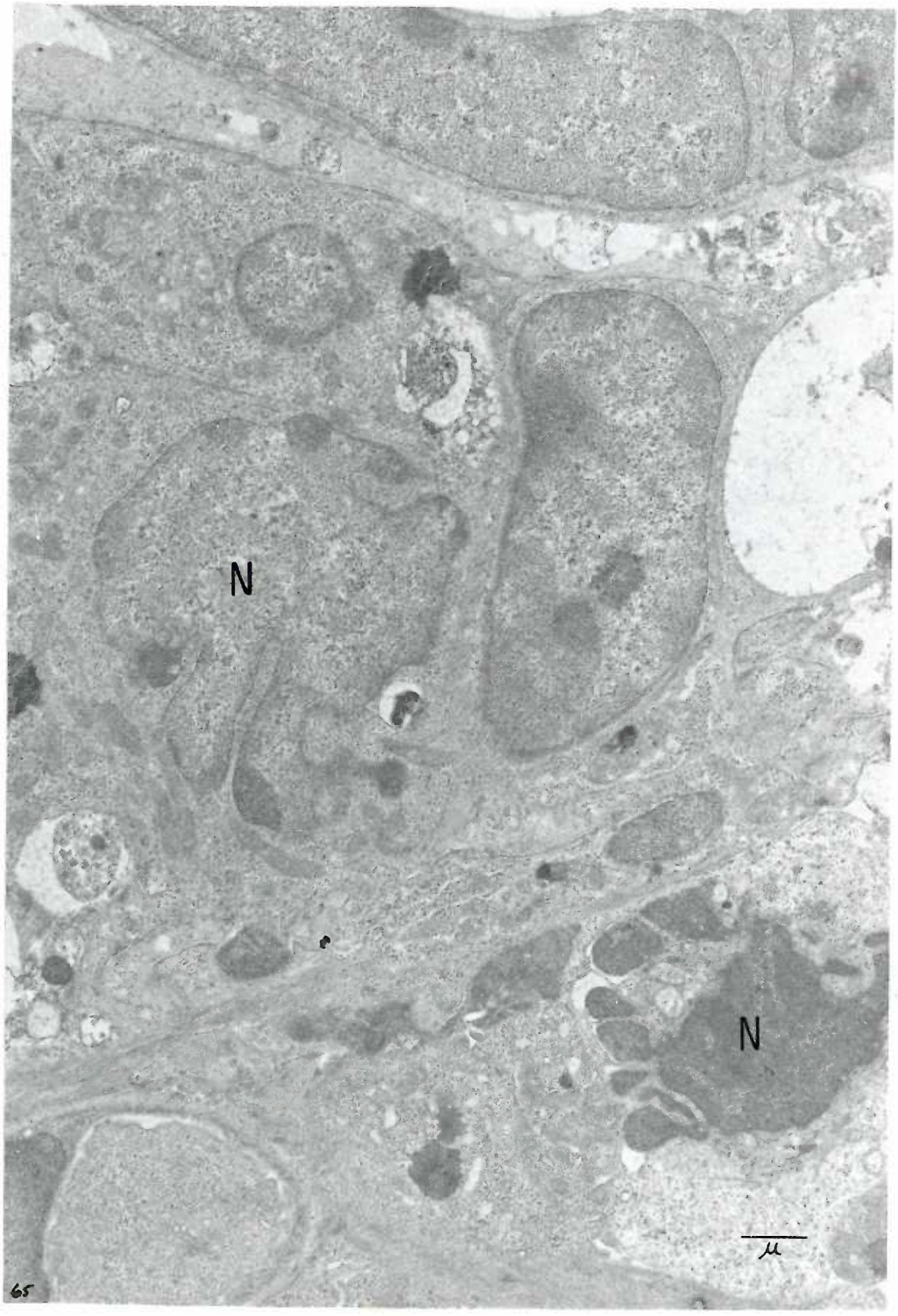
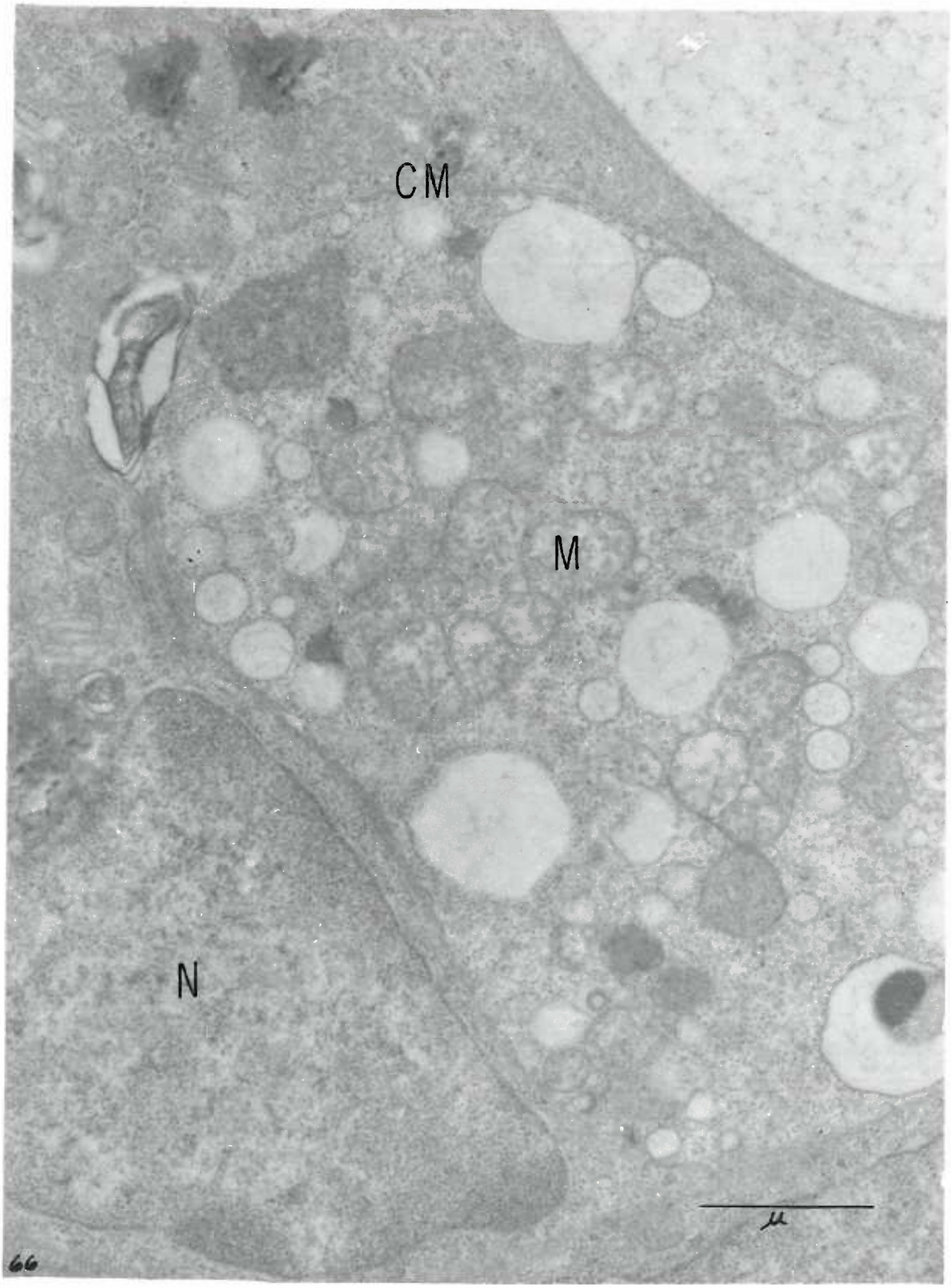

N
N
μ
65

Figure 66. Mouse mammary adenocarcinoma. Cultured in medium 199 with 300 micrograms per ml. of testosterone for one day. Part of degenerating cell in center with cell membrane (CM), relatively empty vacuoles, and swollen mitochondria (M) with fragmented cristae. 28000X.



66