A STUDY OF THE CULTURAL CHARACTERISTICS OF BREAST CANCER, WITH SPECIAL REFERENCE TO ELASTOSIS AND THE PRINCIPLE OF PERIPHERAL FIBROGENESIS IN EPITHELIUM

Thesis submitted for the degree of

Doctor of Philosophy

in the Faculty of Medicine of the

University of Edinburgh

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May 1981

ABSTRACT OF THESIS

Approximately 20% of all breast cancers show a high degree of elastosis and this type of carcinoma has been shown to be associated with a better prognosis.

The work presented in this thesis was undertaken to determine whether a significant difference existed between the cultural characteristics of cells from highly elastotic breast tumours and those of other breast cancers.

Fifteen highly elastotic tumours were cultured out of a total of fifty five breast cancer specimens grown. The cells were examined by phase contrast and scanning electron microscopy and a variety of histological techniques was also employed.

The highly elastotic group emerged clearly as a biological and morphological entity and the production of elastin, by tumour cells, was demonstrated using established histological techniques.

The successful growth of these cells in culture provides a new research tool for further investigation of the highly elastotic tumour.

Seventeen non-malignant lesions and two samples of 'normal' breast tissue, one at 18 weeks gestation, were also cultured in the earlier stages of the work to provide a broad base for interpretation.

A common observation in all the tissue cultures was the peripheral spindling of epithelial cells together with coincident refringent 'halo' formation. Application of standard histological techniques identified the 'haloes' as deposits of reticulin.

These new observations indicate that at least some of the connective tissue in a tumour is tumour cell derived.

Theories/

Theories regarding the "stromal reaction" to a tumour have been discussed in the light of these findings.

In the course of the study cells of many different morphological types were recognised and functional apocrine secretion of mammary carcinoma cells was observed.

A morphological similarity was noted between apocrine and elastotic cells and the possible importance of oestrogen, in connection with both cell types, was raised.

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THE PURPOSE OF THE STUDY

THE PURPOSE OF THE STUDY

Breast cancer is the malignancy which currently claims the largest number of female lives in Britain in any one year and, despite improvements in the diagnosis and treatment of the disease, there is no evidence to suggest that the mortality rate is changing. Any feature of the disease which may assist in its management is therefore worthy of further investigation.

As long ago as 1896 Beatson [14] demonstrated that removing the ovaries of a woman suffering from advanced breast cancer could lead to regression of the tumour. He derived his experiment, rather obliquely, from the observation that castration prolonged lactation in the cow. Although his demonstration has since been confirmed, it is only in one third of breast carcinoma cases that ovarian ablation will be of benefit and, until recently, there was no way of predicting which cases would respond to the treatment.

In 1972 Shivas and Douglas [150] published a paper in which an unequivocal correlation was demonstrated between the quantity of elastica found within a breast carcinoma and the subsequent outcome of the disease. In a series of 103 patients which they studied they showed that patients who had a tumour which contained no elastica whatever survived post-operatively for an average of 33.5 months whereas the mean survival for those patients whose tumours demonstrated a gross degree of elastosis was nearly 94 months, approximately three times as long.

In the same study Shivas and Douglas also considered a smaller series of 50 patients presenting with advanced metastatic disease who, in addition to treatment by mastectomy, had also been treated by adrenalectomy. Once again those patients whose tumours exhibited a gross degree of elastosis survived very much longer, this time approximately/

approximately four times as long as those whose "elastica index" was nil.

More recently Masters et al[105] in 1979 and Millis[110] in 1980 demonstrated a correlation between the response of advanced breast cancer to endocrine therapy and the degree of elastosis of the tumour. They showed that tumours with a high degree of elastosis were more likely to respond to endocrine ablation. They further showed that if the degree of elastosis was correlated with the oestrogen receptor status of the tumour an even more accurate prediction of the response to endocrine therapy could be made.

From these three pieces of evidence alone it seemed that the association of elastica with breast cancer was a phenomenon which warranted further research.

In 1974 Douglas and Shivas[41] had obtained evidence, using the transmission electron microscope, that the elastin originated within the tumour cells and was therefore a tumour product. Further evidence to support this thesis was provided in 1980 when McCullagh et al[109], using immunofluorescent techniques, demonstrated the presence of newly synthesised elastin on the surface of neoplastic mammary epithelial cells.

The purpose of this present study was to examine the growth and behaviour of breast cancer cells using the techniques of tissue culture in the hope that this might help to elucidate the mechanisms of elastin production and perhaps contribute to an understanding of the relatively indolent nature of the highly elastotic tumour.

REVIEW OF THE RELEVANT LITERATURE

a) INTRODUCTION - A NEW APPROACH TO THE INTERPRETATION OF THE GROWTH AND BEHAVIOUR OF BREAST CANCER

Histopathological dogma has long decreed that neoplastic breast disease is an uncontrolled growth of the epithelial cell component of the involved tissue, together with a "stromal reaction" to this growth which is mounted by the normal connective tissue elements in the area of origin of the neoplasm [175].

The aim of any worker attempting to grow human breast cancer in tissue culture has therefore been to succeed in growing a pure population of epithelial cells, free from any contamination by the assumed non-neoplastic, connective tissue producing, fibroblast cell population which may be derived from the "stromal" component.

However, Douglas and Shivas [41] and McCullagh et al [109] have obtained evidence that it may be the tumour cell population which produces the connective tissue elastica in some breast carcinomas and other workers have demonstrated the production of collagen and its precursors by neoplastic mammary epithelial cells [4, 35, 68, 94, 142].

Detailed study of the relevant literature also reveals a number of accounts of cells of one characteristic morphology changing or transmuting to another while under observation, [6, 39, 43, 63, 85, 111, 123, 127, 146, 147.] although the significance of these observations has rarely been commented upon.

The inference from these findings may be that in malignancy there is, in effect, only one cell population although morphological differences may still persist. This view would help to explain some of the difficulties previous workers have had in interpreting their results.

A/

A full appreciation of this altered approach to the interpretation of the growth and behaviour of human breast cancer requires a complete survey of the literature.

b) THE GROWTH OF MAMMARY CARCINOMA IN TISSUE CULTURE

The growth of mammary carcinoma in tissue culture has proved notoriously difficult and a great deal of the literature is concerned with documenting the problems and failures of early workers in the field. It is perhaps pertinent, before considering the growth of breast tissue 'in vitro', to consider the development of tissue culture itself.

TISSUE CULTURE - THE BEGINNING

The dawn of true tissue culture occurred around the turn of the century, although there were at least two reports of cells surviving 'in vitro' before that time. In 1885 Wilhelm Roux[138] explanted the medullary plate of a chick embryo to warm saline where it survived for a few days and in 1887 Arnold[7], having implanted fragments of elder pith into the peritoneal cavity of frogs until they became invaded by leukocytes, removed the fragments to warm saline and subsequently observed the leukocytes migrating and surviving for a short time. In 1897 Loeb[98] managed to maintain the cells of blood, connective tissue and some other tissues from the guinea pig outside the body using small tubes of serum or plasma and in 1898 Ljunggren[97] made a successful transplantation of human skin that had been kept alive for some weeks in ascitic fluid.

In 1903 Jolly[81] succeeded in maintaining salamander leukocytes in a hanging drop for a month and three years later, in 1906, Beebe and Ewing[15] reported that they had managed to cultivate an infectious canine lymphosarcoma in blood from resistant and susceptible animals. Finally, in an experiment which is generally accepted as marking the beginning of true tissue culture, Harrison[69] succeeded in growing recognisable frog axones in lymph clots in 1907.

The/

THE DEVELOPMENT OF TISSUE CULTURE

The demonstration, by Harrison, of the connection between nerve fibres and nerve cells fired the imagination of the research workers of the time and a "rash" of publications on tissue culture soon followed.

Burrows[18] and Carrel[22] were among the first to apply the method successfully to the tissues of warm blooded animals. They used a hanging drop method, with fowl plasma or serum as a medium, for the growth of tissues from the embryo chick.

Carrel and his co-workers also undertook investigations into the composition of the medium and this work, and the work of many other researchers, has led to the development of the synthetic media in use today.

THE GROWTH OF TUMOUR TISSUE

The first attempt at the cultivation of human tumours was by Carrel[23] and Burrows in 1910 and 1911. These two workers, using a plate culture technique and a plasma based medium claimed to have grown a fibrosarcoma and a cancer of the breast 'in vitro' for a few days, but the claim was not supported by illustrations.

In 1914 Losee and Ebeling[99] kept human sarcomatous cells growing in human plasma for more than two months and Fischer[47], in 1927, working with Ehrlich's mouse carcinoma, reported the maintenance of a continuous culture for more than 13 years.

It was not until 1952 that the first permanent human cell line was reported. In that year Gey, Coffman and Kubicek[59] reported the establishment of the, now famous, HeLa cell line from an epidermoid carcinoma of the cervix.

THE GROWTH OF HUMAN BREAST CARCINOMA

In 1936 the first account of the successful growth of cells from human mammary carcinoma appeared. In that year, Gey and Gey[58], using large lying drop slide cultures, reported the growth of 'fibroblast-like' cells[46] from the 'stroma' of four tumour samples.

One year later, in 1937, Cameron and Chambers[24], using the double coverslip technique of Maximow[101], grew both epithelial and fibroblast-like cells from 8 primary breast carcinoma specimens. They also claimed to have witnessed acinus formation, analagous to that seen 'in vivo', 'in vitro'. They did not, however, record the number of attempts made to achieve eight successful cultures.

In 1942 Coman[33], using roller tubes and plasma clots, cultured both benign and neoplastic breast tissue and reported epithelial outgrowths in some, but not all, of the carcinomas cultured. Royle[139], in 1946, also achieved epithelial and fibroblastic outgrowths in all three breast carcinomas which she grew using roller tubes and hanging drops.

In 1952 Pomerat[130] alluded briefly to success in the growth of 'spindle shaped cells' from a biopsy of a scirrhous carcinoma of the breast and in 1954 and 1955 Orr and McSwain[120,121], using plasma clots, Carrel flasks and roller tubes, described sheets, cords and tongues of epithelial cells which, together with fibroblasts, migrated from the breast carcinoma specimens they cultured. In no case did they obtain pure epithelial growth nor did they report a high success rate. Approximately one third of their experiments yielded only poor growth or liquefaction of the plasma clot by the tissue explant. This problem had been noted by many of the earlier workers.

THE 'SPILLAGE' TECHNIQUE

In 1958 Lasfargues and Ozzello[85], observing that neoplastic cells tended to fall out of breast carcinoma specimens when they were cut, and in an attempt to eliminate the fibroblast contamination which had occurred in all previous attempts, reported a technique which has become adopted, with a variety of modifications, by the majority of workers undertaking the growth of breast cancer in tissue culture. Christened the 'spillage' technique, it utilised the fact that epithelial cells were released from the fibrous connective tissue of the tumour when sliced. In this way a pure collection of cells of epithelial morphology was obtained.

CELL LINES DERIVED FROM MAMMARY TISSUE

The Lasfargues and Ozzello paper[85] contained the first account of an epithelial cell 'line' - that is a cell culture which has the potential to be subcultured indefinitely, derived from breast tissue. This cell line, designated BT 20 was derived from a ductal carcinoma of the mammary gland. Although the cell line was isolated and grown initially as an epithelial line the authors reported in their paper that by changing the composition of the medium in which the cells were growing it was possible to induce morphology of a 'fibroblast-like' type and that, with a reversion to the original medium the epithelial appearance of the cells could be regained.

The next report of an epithelial cell line came from Russia in 1963 when Dobrynin[39], using a cell mince and a plasma clot technique, obtained long term growth of the cell line designated CaMa. Once again, as with Lasfargues and Ozzello's cell line, the epithelial morphology was not immutable as this line was originally isolated in fibroblast form and after several months transmuted to epithelial morphology.

In/

In 1965 Foley and Aftonomos[51] tried using collagenase to degrade the connective tissue of a breast carcinoma and so release the tumour cells. This technique had previously been tried unsuccessfully by Lasfargues and Ozzello[85]. Like many of the workers before them Foley and Aftonomos grew both epithelial cells and fibroblasts but failed in their declared aim to establish a cell line of epithelial morphology.

Whitescarver[174], in 1968, conscious of the distinct lack of success experienced by previous workers, entitled his paper 'Problems involved in culturing human breast tissue'. He went on to describe his own attempts, using a variety of techniques, together with the problems encountered. Whitescarver came to the conclusion that treatment of breast tissue with an enzyme would produce fibroblast-like cells whereas the use of Lasfargues and Ozzello's 'spillage' technique would lead to the growth of predominantly epithelial-like cells. Like the majority of workers before him, however, he was unsuccessful in subculturing epithelial cells.

THE USE OF BREAST CARCINOMA CELL LINES GROWN IN TISSUE CULTURE

Whitescarver's paper was followed in 1969 by a paper by Martorelli, Parshly and Moore[103] which described, in passing, the establishment of a line of breast cancer cells from a scirrhous carcinoma using the 'spillage' technique. The purpose of their paper was primarily to describe the effects of chemotherapeutic agents on their cell line and on the BT 20 cell line of Lasfargues and Ozzello. In putting their emphasis on the use of a mammary carcinoma cell line rather than on the problems and techniques involved in growing the cells their paper might have marked the beginning of a new era in the history of the growth of breast cancer cells 'in vitro'.

Accounts/

Accounts of attempts to grow pure epithelial cells from breast tissue still continued to appear, however, and a paper was published in 1970, by Gewant and Goldenberg[60] in which the authors claimed virtually 100% success in obtaining epithelial growth using either an organ explant or a cell mince technique. The illustrations of epithelial cells were, however, not entirely convincing.

BREAST CANCER IN TISSUE CULTURE - AN EXPLOSION IN INTEREST

From 1972 onwards there was an explosion of interest and activity in the field with the publication of numerous papers detailing improvements in techniques and further development of the media used for culturing both normal and neoplastic breast tissue.

The research progressed along many pathways and although some workers now succeeded where their predecessors had failed, managing to establish cell lines of mammary tissue by the methods already described, [11, 72, 87, 117, 129.] by 1972 other methods for growing human breast cells had begun to emerge.

THE GROWTH OF CELLS HARVESTED FROM BREAST FLUIDS

One of the first new methods for growing breast cells in culture was reported in 1972 by Buehring[19]. He succeeded in growing normal human mammary epithelial cells by culturing those cells which could be collected by centrifugation of human breast milk.

Furmanski and his colleagues[54, 144] soon discovered that the number of cells in breast fluid increases dramatically at weaning and in 1974 and 1975 they reported considerable success in growing normal human epithelial cells, using the cells present in post-weaning breast fluids to initiate their cultures.

Reports of work on normal breast cells cultured from breast fluids have/

have continued to appear in the literature [29, 56, 73, 83, 145, 159, 160.] One worker, Buehring[21] extended the use of this technique to the culture of cells originating from breasts containing atypical lesions; he did not, however, use the technique for the culture of malignant cells.

CELL CULTURES FROM PLEURAL EFFUSIONS

Having found that cells could be grown successfully from the naturally occurring cell suspensions of human breast fluids it was a small step to attempt the culture of cells in another "suspension", the pleural effusion of a patient with metastatic breast disease. In 1972 Vasquez[165] and her colleagues reported briefly on the successful growth of cells from the pleural effusion of a woman with metastatic breast carcinoma. By 1973 this culture had been established as a stable cell line designated MCF-7.

Others soon followed; Relda Cailleau and her co-workers succeeded in establishing four cell lines from pleural effusions and they reported their successes in 1974 [25,179]. A review paper in 1978[44] listed no fewer than 22 cell lines, reputedly of breast carcinoma cells, all derived from malignant pleural effusions.

ORGAN CULTURES

To many workers, interested in the action of hormones on the human mammary gland, disruption of the tissue into single cells and subsequent growth as "unnatural" flat sheets of cells was undesirable. These workers investigated small fragments of tissue maintained in organ culture, taking measures to prevent migration of the cells from the cell mass. They then determined the effects of their experimental conditions on the condition of the cells by subsequent fixation and histological or biochemical examination.

The first account of mammary tissue which had been maintained

in organ culture appeared in 1964 when Barker, Fanger and Farnes [10] reported that the addition of insulin to slices of normal mammary tissue, maintained 'in vitro' for up to 12 days, resulted in marked morphological changes of the ductal epithelium including proliferation, hypertrophy and foci of growth resembling squamous metaplasia.

Wellings and Jentoft [170] in 1972, maintained 52 explants of human breast biopsy tissue in organ culture and they also studied the effects of added hormones on the survival of the architecture of the tissue. They reported that, although normal tissue and various dysplastic and neoplastic lesions would survive well without the addition of hormones, scirrhous carcinoma was difficult to maintain. In contrast to Barker et al [10], Wellings and Jentoft found that the addition of the hormones insulin, aldosterone and ovine mammatrophin appeared to have no effect on survival and caused no cell proliferation or secretion.

Flaxman and his co-workers [49,50] also studied the effects of hormones on the maintenance of mammary tissue in organ culture and their work and the work of others has contributed much to the knowledge of the conditions required for the growth of breast tissue in culture.

THE USE OF "NUDE" MICE

Another approach to the maintenance of human breast tissue in culture has been the use of the immunologically deprived, athymic "nude" mouse as the culture vessel.

In 1974 Ozzello[126] and his co-workers transplanted their cell line BT 20 to nude mice where it grew well forming tumours of a similar morphology to the original biopsy. In 1975 Outzen and Custer [122] reported that they had successfully grown three dysplastic and one neoplastic human breast lesion in the cleared mammary fat pads of nude mice. The authors of the paper succeeded in "passaging" one/

one of the dysplastic lesions through four generations of nude mice and the carcinoma was passaged through five mice.

By 1980 this technique had been further applied as a means to derive three cell strains from two transplantable human breast tumours established in the nude mouse.[137]

c) EPITHELIAL CELLS AND FIBROBLASTS

The foregoing survey illustrates the variety of methods which have been used to try to grow human breast tissue in culture. Because it has always been considered to be the epithelial component of a tumour which is the malignant entity and the connective tissue component or "stroma" which is the host's "reaction" to the tumour tissue, the goal of all research workers growing mammary carcinoma in culture has been to obtain a culture of pure epithelial morphology. For this reason, many workers have discarded those cultures which contained large proportions of spindle shaped "fibroblast-like" cells. However, as alluded to briefly earlier (pp 3), there are many instances quoted in the literature of an apparent transformation from one morphological cell type to another and often the cells seem capable of reversion to their original form on modification of the culture conditions.

CHARACTERISTICS OF EPITHELIAL CELLS IN CULTURE

Epithelial and "epithelial-like" [46] cells in culture are those cells which tend to form sheets of closely adherent polygonal forms. Transmission electron microscopy of normal and neoplastic mammary epithelial cells in culture [20, 48, 124, 125, 144, 145] reveals a wide spectrum of ultrastructural features. Those which are taken to be characteristic of the epithelial component of the breast tissue include the presence of oval, sometimes indentate nuclei with evenly distributed chromatin and frequent, occasionally very large, nucleoli. There are usually numerous free ribosomes distributed through the cytoplasm together with the endoplasmic reticulum which is rough surfaced. The Golgi complex is inconspicuous in mammary epithelial cells and the mitochondria are usually few and irregularly distributed. Various other cellular inclusions; glycogen/

glycogen granules, lysosomes and fine filaments may also be found in the cytoplasm. One of the features which is considered to be most characteristic of epithelial cells is the presence of the intercellular contacts; desmosomes and junctional complexes.

CHARACTERISTICS OF FIBROBLASTS IN CULTURE

In contrast to the polygonal epithelial cells, fibroblasts and "fibroblast-like" cells[46] are cells of spindle or irregular shape. These cells characteristically have an elongated nucleus with no noteworthy ultrastructural features[124]. Fibroblasts growing in culture exhibit the phenomenon of contact inhibition[1] and tend to line up in parallel rows. There are no cell contacts such as desmosomes between true fibroblasts and, as the name implies, these cells are the cells believed to be responsible for fibre formation[46].

MORPHOLOGICAL CELL TRANSFORMATION 'IN VITRO'

In 1913 Champy[30] described the morphological "transformation" of cultures of foetal rabbit epithelium to connective tissue cells. He explained this phenomenon by postulating a process of "dedifferentiation" of epithelial cells to the more "undifferentiated" connective tissue cells.

Uhlenhuth[163, 164], in 1914, working with epithelial cells from the frog, referred to "spindle-shaped epithelial cells" which he saw at the periphery of epithelial islands of tissue when they were grown in a semi-solid medium.

W. C. Clarke[32], in 1915/16, in an experiment designed to determine the origin of the regenerated lining of damaged internal body surfaces, postulated that exposed connective tissue cells proliferate, change in form, flatten and become epithelial in nature. He cited two examples of morphological changes 'in vitro' when he described experiments which were/

were conducted on chick embryo cells and sarcoma cells from a St. Bernard dog which were cultured in plasma clots in an "observation incubator".

In 1922 Fischer[47], while reporting the establishment of a pure strain of epithelial cells from the lens tissue of embryonic chicks, described cells at the periphery of well established epithelial cultures which had a "spindle" shape and resembled fibroblasts. He explained the presence of these cells by proposing that the shape of the cells was a reflection of the mechanical conditions prevailing. This was essentially the same supposition as that made by Uhlenhuth[163, 164] but Fischer found the direct opposite of Uhlenhuth; the fusiform cells appeared at an area of dense medium - the edge of the clot, not in the soft medium towards the centre of the clot.

Warren Lewis[88, 89], in 1923, gave a graphic description of a transformation of cell form in his reports on the visible transformation of a loose network of embryonic chick heart mesenchyme to a flat "mesothelial" membrane.

Henle and Dienhardt[77], in 1957, while establishing five normal human cell lines, found that in all cases an initial outgrowth of fibroblasts and "histiocyte-like" cells was obtained. After 60 to 92 days, however, new, epithelial cell types appeared and these gradually became dominant so that all cell lines established were of epithelial morphology. Also in 1957, Lasfargues[84], describing the cultural behaviour of the normal mammary epithelium of the adult mouse, gave an account of "some of the most peripheral cells, showing a particularly strong pseudopodial activity may wander away taking on a fibroblastic appearance"

Lasfargues and Ozzello[85], in their paper reporting the "spillage" technique for the cultivation of human breast carcinomas described how, by/

by altering the proportion of mucopolysaccharides present in the growth medium used, they could alter the morphology of the cells. The cells grown, later designated cell line BT 20, were originally isolated as "epithelial-like" cells using mucopolysaccharide rich umbilical cord extract. If cord extract was then removed from the medium the cells elongated and became polarised to take on a typical "fibroblast-like" morphology. By altering the medium once again and reverting back to the original cord extract rich medium, the epithelial morphology could be regained.

In 1961 Sanford, Dunn and Westfall[147], working with cultures of mouse mammary carcinomas, found that prolonged maintenance of the cells 'in vitro' could lead to a change in their character when retransplanted into a mouse. They found that cells which five months previously had produced a carcinoma on retransplantation subsequently gave rise to a sarcoma.

Another report of a possible morphological transformation appeared in 1963 when Dobrynin[39] reported the establishment of the cell line CaMa. In contrast to the Lasfargues and Ozzello cells, these were originally isolated in fibroblast form and it was only after several months in culture that a change to epithelial morphology occurred.

In 1965 Willmer[177] drew attention to "The Problem of Interconversion" in a chapter in the book "Cells and Tissues in Culture". In this work Willmer discussed an intriguing theory which he had put forward in 1960[176] - all cells originate as "epitheliocytes" when they surround the hollow blastula-like organism which is an integral part of the evolution of all metazoan animals. In this way an epithelial cell could be considered to be the most primitive form of metazoan cell and any change to a fibroblast-like cell would be looked upon as differentiation. Conversely, the/

the change from a fibroblast to an epithelial cell would be considered "de-differentiation". This view was in direct opposition to that put forward by Champy[30] in 1919 and underlines a profound uncertainty as to the "direction" of differentiation.

A slightly different, but well documented change in cellular morphology 'in vitro' has been described by Ozzello and others[123, 127] in their accounts of cell growth from histiocytomas, fibrous xanthomas and dermatofibrosarcoma protruberans. Cells from all these lesions were observed growing in culture and a change from a histiocytic form to a fibroblastic form was described.

MORPHOLOGICAL CELL CHANGE 'IN VIVO'

It is not only 'in vitro' that cells have been noted to change their form. Apolant[6] and Ehrlich[43], in 1905, described how the character of a mouse mammary tumour changed from a carcinomatous form to a sarcomatous form over the period spanned by approximately 9 generations.

In 1945 Ludford[100] noted a similar change in murine mammary tumours but attributed the change to sarcomatous transformation of the "stromal reaction" to the original tumour. Goldfeder and Nagasaki[63], in 1954, also recorded a change from carcinoma to sarcoma in a mouse mammary tumour although they concluded that the change was in the epithelial component of the tumour and not in the surrounding stroma.

In 1956 Leighton, Kline and Orr[90] grew normal human foreskin fibroblasts which, after 23 months in culture, transformed to histologically malignant cells. The published photograph of these malignant cells looks remarkably like a carcinoma, that is - malignant epithelial cells.

THE PRODUCTION OF CONNECTIVE TISSUE COMPONENTS BY EPITHELIAL CELLS

The well documented accounts of gross morphological change from one cell type to another could imply that a change in cellular function might also/

also occur. Although no investigations correlating change in morphology with a change in function have been reported there are several accounts in the literature of morphologically "epithelial" cells producing connective tissue elements - a property usually ascribed solely to the fibroblast and closely related cells such as the osteoblast.

The first clear demonstration of collagen production in tissue culture, by cells other than fibroblasts, came in 1965 when Green and Goldberg [66] showed that HeLa cells, although not of connective tissue origin, did possess the capacity to synthesise collagen. The technique by which they demonstrated this phenomenon involved measurement of the incorporation by the HeLa cells of labelled proline into the hydroxyproline residues of collagen. The extent to which HeLa cells demonstrated this capacity was approximately equal to that shown by the fibroblast cell line 3T6 when the latter was in exponential growth - the growth phase showing the lowest collagen production.

Gonzales-Licea [64], in 1967, while studying a form of breast cancer in which bone formation is a notable feature, found evidence, using the transmission electron microscope, not only of bone formation by the undifferentiated tumour cells but also of the capacity of these cells to produce collagen.

Dodson and Hay [40, 71], in 1971 and 1973, demonstrated the production of collagen by an embryo chick corneal epithelium when they showed that radioactivity, introduced to the cultures in the form of ^{3}H-proline, was associated with striated fibrils and sheets of material which had the interband pattern typical of native collagen.

As mentioned previously (pp 2), Douglas and Shivas [41] (1974) found evidence of connective tissue production by neoplastic epithelial cells/

cells when they located both elastic fibrils and collagen fibres in transmission electron micrographs of breast carcinoma cells. Another paper by Shivas, this time in collaboration with Mackenzie[151], lends further support to the thesis that neoplastic epithelial cells can secrete their own collagenous matrix. As a "follow up" to the work on the production of elastica by tumour cells, Shivas and Mackenzie took six scirrhous carcinomas of the breast and studied the areas of typical scirrhous growth using the transmission electron microscope. They found a complete absence of both the formed elements and the cells of normal connective tissue. Collagen fibrils were mixed with small quantities of elastica together with an indeterminate matrix which seemed to emanate from the tumour cells. Shivas and Mackenzie concluded that "the ultrastructural evidence supports the conclusion that "stromal reaction" in scirrhous carcinoma of the breast is a product of the tumour cells".

Also reported in 1974 was a continuation of the work of Green and Goldberg[66] on the synthesis of collagen by cell lines in culture. The two workers involved, Langness and Udenfriend[86] found, like their predecessors, that there was evidence of collagen production in cell lines of non-fibroblastic origin. These workers put forward the suggestion that - "fibroblasts are not as central to the process of collagen formation as we have been led to believe".

In 1975 Al-Adnani, Kirrane and McGee[4], while studying scirrhous carcinomas of the human breast, found, using two immunohistochemical techniques, that the malignant epithelial cells in 30 out of 32 of the tumours they studied contained not only collagen but also its active precursor, prolyl hydroxylase. They further found that neither the enzyme, nor collagen were detectable in the spindle cells of the "stroma" of/

of the tumour and they suggested that the malignant epithelium of scirrhous breast carcinoma produces its own collagenous stroma and that the scirrhous "reaction" in these tumours is not a host response to tumour invasion but a tumour product.

Groniowski, Walski and Pietrov [68] came to a similar conclusion in their report, published in 1975, in which they described fibrils, detected using the transmission electron microscope, in the cancer cells of two breast tumour biopsies. This work followed on from their earlier paper in which they had described the presence of striated collagen fibrils in the hyaloplasm of human hepatocytes.[67]

In 1976 Hay [93], in collaboration with two other workers, published an expansion of her original work on the secretion of collagen by embryonic chick corneal epithelium. Once again they labelled the collagen synthesised with tritiated proline and, after analysis, they concluded that the majority of the collagen synthesised was Type I, the type found in bone, tendon and skin, with a small amount of Type II, as found in hyaline cartilage and vitreous humour.

In 1978 Roesel, Cutroneo, Scott and Howard [142] reported a study on collagen production by cloned mouse mammary tumour cells. They found that these, too, were capable of collagen production as judged by the activity of prolyl hydroxylase in the cultures.

In 1979 a group of workers headed by Liotta [94] found, using an immunoperoxidase technique, that neoplastic mammary epithelium is capable of synthesising Type IV collagen, the type of collagen which is taken to be synonymous with basement membrane.

Thus most of the connective tissue components have been found to be produced by either normal or neoplastic epithelial cells.

The/

THE PRODUCTION OF CONNECTIVE TISSUE BY OTHER NON-FIBROBLASTIC CELLS

It is, perhaps, less surprising to find that cells such as smooth muscle cells and endothelial cells can produce connective tissue components 'in vitro' since, embryologically, muscle cells, endothelial cells and connective tissues are all thought to be derived from the mesoderm.

In 1967, in an editorial devoted to the arterial medial cell, Wissler[178] makes the assumption that there is a single cell type in the arterial media "a cell which is probably responsible for the production of collagen, elastin, smooth muscle fibres and basement membrane". Wissler feels that this single, multifunctional medial cell is most likely, morphologically, to be the smooth muscle cell and he therefore gives a central role to this cell in the production of structural connective tissues.

Ross[140], in 1971, lent support to Wissler's assumptions by finding that the smooth muscle cells from the intima of a guinea pig aorta would secrete microfibrils with the characteristic composition of elastic fibres when the cells were grown in tissue culture. In collaboration with Glomset, Ross[141] showed that smooth muscle cells from yet another species would synthesise elastic fibre proteins. In 1973 these authors took smooth muscle cells from the medial layer of the thoracic aorta of Macaque monkeys and found that, when maintained in culture for about two weeks, the cells would produce elastic fibre proteins and collagen.

In 1978 Foster[52], together with a number of other workers, concluded that rabbit aorta smooth muscle cells, which were maintained in tissue culture, were synthesising a form of elastin which could be "chased" biochemically into the insoluble elastin precursor - tropoelastin.

Endothelial cells have also been shown to be capable of the production of connective tissue components.

Jaffe[80], in 1976, demonstrated the production of elastic tissue microfibrils/

microfibrils and elastin fibres in transmission electron micrographs of the matrix of cultured human umbilical vein endothelium. The cells they cultured also appeared to synthesise basement membrane in an amorphous form.

Another paper published in 1976 also implicated endothelial cells in the production of Type IV collagen or basement membrane. Howard, Macarak, Gunson and Kefalides[78] cultured calf aorta endothelial cells and detected the formation of basement membrane by the incorporation of labelled proline and lysine. They confirmed that the type of collagen produced was Type IV by binding a fluorescent anti Type IV antiserum to the newly formed moieties.

Hart, Beydler and Carnes[75] came to the conclusion that arterial endothelium was probably the source of intimal elastin when they studied the structure of the pig thoracic aorta using the scanning electron microscope. They stated "the presence of a very thin subendothelial layer of delicate fibrils, differing from the medial fibres, suggests that the normal intimal elastin may be a product of endothelial cells".

Barnes, Morton and Levene[13], in 1978, while culturing pig aortic endothelial cells, demonstrated Type I and Type III collagen production. The closing paragraph of their paper serves to underline the uncertainty as to the origin of connective tissue components: "The synthesis of interstitial collagens by endothelial cells in culture implies no simple distinction can be drawn between cells of epithelial type producing basement-membrane collagens (IV & V) and the fibroblast and its differentiated forms producing interstitial collagens (I, II & III). Similarly smooth muscle cells have been shown to synthesise α (A) and α (B) chains[104] as well as Type I and Type III collagens, whilst embryonic/

embryonic chick corneal epithelium has been shown to synthesise interstitial collagen[40, 71]."

Accounts of connective tissue production by endothelial cells have continued to appear. 1979 saw the publication of a paper by Jones[82] in which he described how he managed to construct an artificial blood vessel wall by growing cloned bovine endothelial cells, which secreted their own basal lamina, on a preformed layer of rat smooth muscle cells.

Bovine aortic endothelial cells were again shown to synthesise a pro-collagen in culture by Sage et al[148] and Carnes, Abraham and Buonassisi[27] have demonstrated the production of a soluble elastin by rabbit endothelial cells.

CELLS OF "INDETERMINATE" TYPE

The supposition that there may, in effect, be only one cell population in malignant, or indeed in normal, tissue is supported by a few accounts in the literature of cells of "transitional" or "intermediate" type. The fact that there are not more accounts of indeterminate cells is probably a reflection of the unconscious selection procedures which may be practised by research workers in their climate of thought arising from the long-established germ layer dogma. If one is expecting to find cells of a particular recognisable morphology, a cell which is difficult to classify might be passed over.

In 1965 Willmer[177], in his treatise on "morphological problems of cell type, shape and identification" stated "it may be extremely difficult to identify particular cells". He cited many examples of cellular shape change 'in vitro' but he did not describe any "intermediate" cells in such terms.

Ahmed[2] and Harris[74], on the other hand, while describing the/

the myoepithelium in malignant breast lesions, found ultrastructural evidence of "intermediate cells showing features of both fibroblasts and myoepithelial cells". Ahmed[2] went on to suggest that myoepithelial cells might transform to fibroblasts in malignant conditions, thus explaining the apparent absence of myoepithelial cells in such situations.

A number of workers have described, in passing, "spindle-shaped epithelial cells" [51, 92, 111, 139] Groniowski[68], in 1975, remarked on "transitional forms between polyhedral and spindle-shaped cancer cells" in his paper putting forward the view that it was the cancer cells which produced the fibrous stroma in breast carcinoma.

Prop, Prop-Arnold and Eijgensten[135], in 1976, while investigating sporadic and hereditary retinoblastoma, described and published photographs of a cell type "intermediate between epithelial and fibroblastic" which migrated from a plasma clot culture of the tumour.

The discussion about a paper by Prop[136] in "Human Tumours in Short Term Culture" (1976) contains a comment by Stoker that in their experience of cultures of breast tissue prepared by the spillage technique, the cells propagated most successfully were neither epithelial cells nor fibroblasts but "intermediate cells".

It is thus clear that "cells are not always what they seem" and it was in the light of the information presented in the Survey of the Literature that samples of breast tissue were taken, set up in tissue culture and subsequently examined.

EXPERIMENTAL SECTION

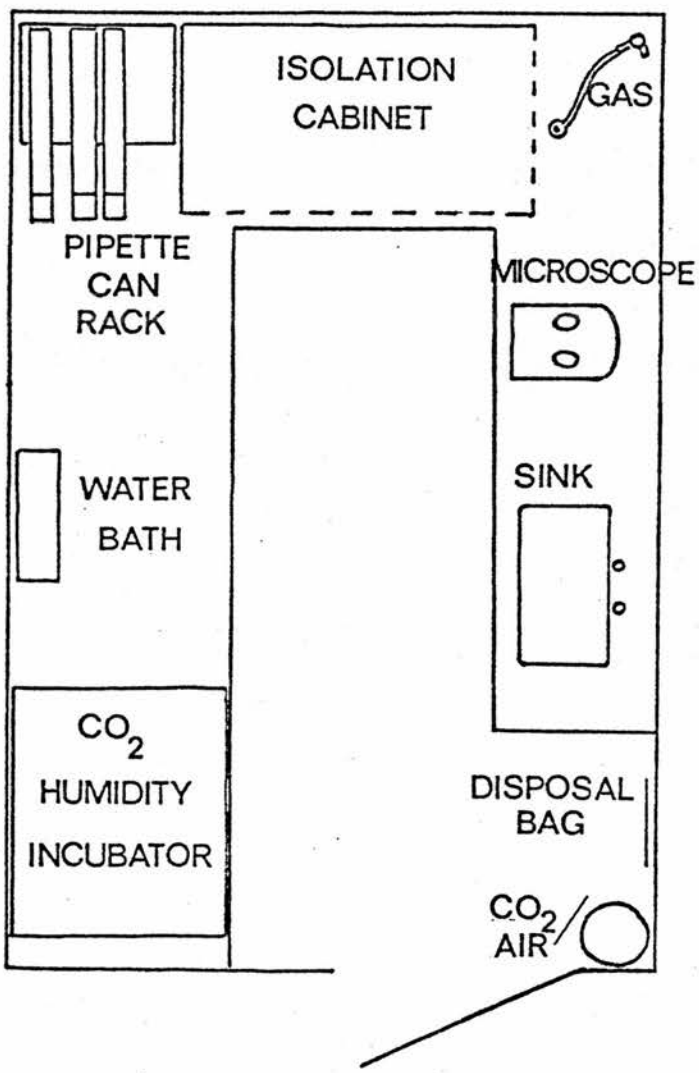
EXPERIMENTAL SECTION

INTRODUCTION

Prior to the initiation of the work for this thesis there was no tissue culture facility at Ethicon Ltd. The first task was, therefore, to set up a tissue culture laboratory.

A room 9 ft. x 6 ft. was converted for use as a tissue culture laboratory, the layout being:-

TISSUE CULTURE LABORATORY



Further facilities included a refrigerator, a deep freeze, a high temperature sterilising oven, an autoclave and irradiation facilities and a washing-up area with a Fi-stream distillation apparatus located elsewhere in the Ethicon Research Unit.

Access to the laboratory was limited to the writer and the tissue culture technician.

PRECAUTIONS

The possibility that viruses may be implicated in human breast cancer has still to be eliminated. In order to minimise any chance of viral infection and also to avoid the chance of infection with any other pathogenic organism which may be present in biopsy tissues, certain precautions were followed while handling the human material.

1. Experimental material was always handled in the isolation cabinet.
2. Experimental material was always handled with sterile instruments.
3. Any instrument or experimental apparatus which had been in contact with the human material was immersed for 24 hours in Cidex activated glutaraldehyde (Arbrook Ltd.) before being subjected to the normal glassware washing-up routine.
4. Disposable items were sealed in impermeable plastic bags and were subsequently incinerated.
5. Old medium was treated with Cidex before disposal.
6. Flasks which had not been histologically 'fixed' with formalin or glutaraldehyde for subsequent processing were sealed in impermeable plastic bags and incinerated.

I. MATERIALS

1. TISSUE

Fresh tissue was collected, within 30 minutes of removal from the patient, from the Frozen Section Laboratory of the Edinburgh Royal Infirmary. The tissue selected from the biopsy or mastectomy specimen was collected in cold Complete medium (see below) and was stored at $4^{o}C$ until processed further. Usually the material was stored overnight.

2. MEDIUM - COMPLETE CULTURE MEDIUM

Complete culture medium was made up by combining:-

100 mls. RPMI 1640 medium (Gibco Europe)

15 - 20% Foetal Bovine Serum, virus and Mycoplasma screened (Gibco Europe)

10 - 20 ug/ml insulin zinc (Wellcome)

100 IU/ml penicillin and 100 ug/ml streptomycin (Gibco Europe)

II. METHODS

1. CELL CULTURE

i) SETTING UP PROCEDURE

The procedure used was essentially the 'spillage' technique described by Lasfargues and Ozzello in 1958[85].

The tissue was removed from the collection medium with sterile forceps and placed in 10 mls of phosphate buffered saline ((PBS) - Dulbecco's formulation, without Calcium and Magnesium - Gibco Europe) in a sterile 10 cm glass petri dish. Any excess fat was trimmed from the tissue with a sterile Ever Ready Single Edge Corrux blade and the remaining tissue was transferred to a further 10 mls PBS in a second sterile petri dish.

The tissue was carefully sliced with a fresh sterile blade to release cells into the PBS. The cells and PBS were then removed to a sterile conical centrifuge tube and, for the first 65 biopsies, allowed to sediment under unit gravity for 15 - 30 minutes. Biopsy specimens 65 - 86 were centrifuged at approximately 300 rpm for 5 minutes. The supernatant was then removed and, if much debris was present, a further quantity of PBS was added to the cells and the sedimentation was repeated. Fresh medium was then added to the resuspended cell pellet and the cells and medium were transferred, in 5 ml aliquots, to Nunclon 25 sq cm surface area tissue culture flasks. The number of flasks set up per biopsy varied according to the turbidity of the cell/medium mixture.

The pH of the cell suspension was adjusted to 7.3 by gassing the cultures with a 95% air : 5% CO_2 mixture (BOC Special gases), the flask caps were tightened and the flasks were incubated at 37°C.

ii) RENEWAL OF CULTURE MEDIUM

The medium above the cells was changed approximately every 7 days,

or/

or more often if it turned yellow indicating a reduction in the pH. Old medium was removed from the flask using a 2 ml capacity sterile disposable transfer pipette (Alpha Laboratories). The old medium was either collected for further examination (see Histology Section p.34) or was discarded following the precautions outlined above.

Fresh Complete medium was added, in 5 ml aliquots, to the flasks which were gassed with the 95% air : 5% CO_2 mixture and they were reincubated at 37°C.

iii) CELL PASSAGE

In some instances growth of the cells was so vigorous that it became imperative to transfer to a larger container, i.e. passage the cells, and thus initiate a 'Primary cell line'. When cell passage was indicated the old medium was removed with a sterile disposable pipette and the cells were washed with two 5 ml aliquots of PBS (Dulbecco's formulation, without Ca and Mg) at 37°C. 5 mls of a 0.25% solution of Trypsin (1/250 Flow Laboratories) in PBS was added and left in contact with the cells for one minute. The trypsin was then removed, the flasks were closed and then incubated for 15 minutes at 37°C. At the end of this period fresh Complete medium was added to the cells, which had been released from the floor of the flask by the action of the trypsin, and the cell/medium suspension was transferred to further flasks. These new flasks were gassed with the Air/CO_2 mixture and reincubated at 37°C.

iv) EXAMINATION OF CULTURES

The cell cultures were examined periodically using an Olympus CK inverted microscope fitted with X 10 and X 20 phase objectives and a phase contrast condenser. A note of the cellular appearance was made and the growth characteristics were recorded photographically.

v)/

v) MYCOPLASMA SCREENING

No attempt was made to screen for mycoplasma contamination owing to the scarcity of the experimental material.

2. PHOTOMICROGRAPHY

TISSUE CULTURE

i) Camera System.

The Olympus inverted microscope was fitted with a Nikon PFM photomicrography unit with an M35 S dark box. The magnification of the camera eyepiece was X 15.

ii) Films

a. Black and White. - Films 1 - 74 were taken on Kodak Pan F film. Filma 75 - 111 were taken on Kodak Technical Pan film, number 2415.

b. Colour. - Colour photographs were taken initially on Kodak Ektachrome 64 Professional colour transparency film. The slides were later transposed onto Kodak Vericolour II Professional film 5025 Type S.

iii) Technical Details.

a. Black and White.

Light intensity for magnification X 10 - 7, for magnification X 20 - 8 on Olympus microscope scale.

Exposure - 2 seconds for plain light.

8 seconds for phase contrast illumination.

b. Colour.

Using a blue filter - for magnification X 10 - light intensity 7, exposure 8 secs.

iv)/

iv) Development and Printing

a. Black and White.

Pan F film was developed with Adox M.Q. Borax.

Technical Pan film was developed with H.C.110, according to the specifications for moderate contrast results.

Photographs were printed on Kodak Veribrom paper, Grade F2.

b. Colour.

Ektachrome 64 Professional film was developed by the Kodak E6 process.

Kodal Vericolor 11 film was developed with the C41 Flexicolor process and photographs were printed on Kodak Ektacolor 78 paper.

HISTOLOGICAL PREPARATIONS

i) Camera System.

An Olympus Vanox microscope fitted with a PM-10A automatic camera system was used.

ii) Film.

Colour photographs were taken on Kodacolor II C135 colour negative film.

iii) Development and Printing

The C41 Flexicolor development process was used.

Photographs were printed on Kodak Ektacolor 78 paper.

3. HISTOLOGICAL TECHNIQUES

i) Fixation and Processing

a) In the flask - Where cells were attached to and growing on the flask floor. Flasks in which cells were to be examined histologically were first washed with two changes of PBS (Dulbecco's formulation without Ca and Mg) at 37°C. The cells were then fixed with neutral buffered formalin.

b) "Droplets" - Cells and debris which were floating in the medium were collected in conical centrifuge tubes and centrifuged at 1000 rpm. for 5 minutes. The supernatant was then removed and discarded and the solid matter in the pellet was resuspended in a very small quantity of complete medium. Drops of this cell suspension were then placed on clean microscope slides where they were allowed to spread and the medium to evaporate. The preparations were then fixed with 100% methanol.

c) Agar blocks - The cells and debris were centrifuged as in (b) and the supernatant was removed. The cell pellet was then "bound" with 2% Agar (Gibco Europe) which was solidified quickly by plunging the tubes into an ice/water mixture. The agar/cell block was removed from the centrifuge tube using a long, narrow glass rod to ease the agar away from the side of the tube. The block was then placed in neutral buffered formalin fixative. The fixed agar/tissue blocks were embedded in wax using standard histological procedures and 5 μm sections were cut, placed on glass microscope slides and stained.

ii) Methacrylate tissue embedding.

The standard of histological sections obtained using resin embedded material is superior to that obtained using wax. Selected samples were therefore embedded in Glycol methacrylate using the following technique:

Solutions

Uncatalysed solution 'A'

Low acid Hema (Glycol methacrylate) Hartung Associates - USA.	160 ml.
2 - Butoxyethanol	32 ml.

The two solutions were mixed and stored at 4°C.

Catalysed Solution 'A'

Uncatalysed Solution 'A'	100 ml.
Benzoyl peroxide	0.9 gm.

The Benzoyl peroxide was mixed with the uncatalysed Solution 'A' and stored in the dark at 4°C.

Solution 'B'

Polyethylene glycol 400	15 ml.
N, N Dimethylaniline	1 ml.

The two solutions were mixed and stored, in the dark, at 4°C.

Final embedding medium

30 parts Catalysed Solution 'A' : 1 part Solution 'B'.

Method

1. The formalin fixed tissue was placed in a Tissue-tek capsule and washed in running water for 1½ - 2 hours.
2. The tissue was dehydrated through graded methylated spirit:
   1 hour in 50% methylated spirit.
   1 hour in 70% methylated spirit.
   1 hour in 80% methylated spirit.
   1 hour in 95% methylated spirit.
   1 hour in 100% methylated spirit.

3./

3. The tissue was stored in fresh 100% methylated spirit overnight. It was then:

4. Infiltrated with a 1:1 mixture of Catalysed Solution 'A' and 100% methylated spirit - 2 X 2 hours.

5. Infiltrated with pure Catalysed Solution 'A' for 3 - 4 hours in a desiccator at 400 mm Hg. pressure.

6. As step 5.

7. Infiltrated with fresh pure Catalysed Solution 'A' under vaccuum, overnight.

8. Preparation of Embedding Mixture.

   30 parts of Catalysed Solution 'A' were mixed thoroughly with 1 part of Solution 'B' at room temperature. The solution was then placed in the base of an embedding mould.

9. The tissue was placed in the base and the embedding solution was "topped-up".

10. A metal block holder was then placed gently in position on the base and air bubbles were allowed to escape. Further drops of solution were added to the central hole in the stub to exclude all air.

11. The edges of the blocks were then sealed with wax and the central hole was sealed with autoclave tape.

12. The blocks were left undisturbed overnight to polymerise.

13. When polymerisation was complete sections were cut using a Jung Autocut microtome fitted with a glass knife.

iii) Staining techniques

Where cells were stained 'in situ' this was done by introducing the required reagent into the flask for the required period and then decanting.

Microscope slides were stained in standard histological containers.

a. Gordon and Sweets Silver impregnation method for Reticulin fibres.

Special reagents:

1) Silver solution. To 5 ml 10.2% aqueous silver nitrate, strong ammonia (.880) was added drop by drop until the precipitate which was first formed just dissolved. 5 ml 3.1% NaOH was then added drop by drop until the resulting precipitate was just dissolved. The solution was made up to 50 mls with fresh distilled water.

2) Acidified potassium permanganate.

95 ml 0.5% Potassium permanganate.

5 ml 3% Sulphuric acid.

Method

1. Wax sections were brought to water. Flasks and droplet preparations were rinsed with tap water.

The preparations were:

2. Oxidised with acidified potassium permanganate for 5 minutes.
3. Washed in water.
4. Bleached with 1% oxalic acid for 3 minutes.
5. Rinsed in distilled water.
6. Washed in tap water and then 2-3 changes of distilled water.
7. Mordanted in 2.5% Iron alum for 10 minutes.
8. Washed in 3 changes of distilled water.
9. Treated with silver solution for 30 seconds.

10/

10. Washed well with several changes of distilled water.
11. Reduced with 10% freshly prepared aqueous formalin for 1-2 minutes.
12. Washed in tap water and then in distilled water.
13. Toned in 0.2% gold chloride for 10 minutes.
14. Rinsed in distilled water.
15. Fixed in 5% sodium thiosulphate for 5 minutes.
16. Washed in water for 1 - 2 minutes.

The flasks were then filled with fresh water and the preparations photographed.

Slide preparations were sometimes counterstained with a very weak solution of neutral red, they were then dehydrated, cleared and coverslips were mounted with DPX.

b. Gomori's Aldehyde Fuchsin for the demonstration of elastic fibres.

Staining solution:

Basic Fuchsin (Polysciences Inc. Basic fuchsin C.I. 42510)	1 gm.
70% IMS.	200 ml.
Concentrated hydrochloric acid	2 ml.
Paraldehyde - fresh (that paraldehyde which is in liquid form when the container has been removed from the refrigerator for a short time.)	2 ml.

The fuchsin was dissolved in the alcohol and the HCl and paraldehyde were then added. The mixture was shaken well and allowed to stand at room temperature until it was deep purple (24 - 48 hours). The solution is stored in the refrigerator and retains its selective staining properties for 2 - 3 months.

Method.

The preparations were brought to water and they were then:
1. Treated with Lugol's Iodine for 10 minutes.

2/

2. Transferred to 5% Sodium thiosulphate for 5 minutes.

3. Washed in running tap water for 3 minutes.

4. Rinsed in 90% alcohol.

5. Stained for 10 minutes.

6. Rinsed in 95% alcohol.

Preparations which were stained in the flask were then bathed in alcohol and photographed.

Slide preparations were counterstained very lightly with Tartrazine or Light green and the sections were then dehydrated and cleared. Coverslips were mounted with DPX.

c. Periodic Acid Schiff technique for the demonstration of Mucopolysaccharides

Staining solution:

Schiff's Leuco-fuchsin solution:

1 gm. basic fuchsin was dissolved in 200 ml hot distilled water and brought to boiling point. It was then cooled to 50°C and filtered. 20 ml N.HCl was added and the solution cooled. 1 gm. anhydrous sodium bisulphite was then added. The mixture was then kept in darkness for 48 hours until it was straw coloured. The staining solution was stored in the refrigerator.

Test for Schiff's Leuco-fuchsin solution:

A few drops of staining solution are added to 10 mls neutral buffered formalin. If the solution turns to a red/purple colour it is still 'viable'. If the solution turns to a blue/purple it is breaking down.

Method:

Wax sections were brought to water and flasks were rinsed with water. The preparations were then:

1. Rinsed with distilled water.

2. Treated with 0.5% Periodic acid solution for 5 minutes.

3./

3. Rinsed in distilled water.
4. Treated with Schiff's Leuco-fuchsin for 15 minutes.
5. Immersed in running tap water for 10 minutes.
6. Treated for 6 minutes with Haematoxylin.
7. Rinsed with tap water.
8. Differentiated in acid alcohol to weak pink.
9. Washed in running tap water.
10. 'Blued' in running tap water.

Material in flasks was then dehydrated with alcohol and photographed beneath absolute alcohol.

Slides were dehydrated and cleared. Coverslips were mounted with DPX.

d. Lillie and Ashburn's Isopropanol Oil Red O method for the demonstration of lipids.

Staining solution:

A saturated solution of Oil Red O (0.25 - 0.5%) in isopropyl alcohol is kept in stock. For immediate use, 6 mls of stock solution is diluted with 4 ml of distilled water and the mixture is allowed to stand for 5 - 10 minutes after which it is filtered and used within 1 hour.

Method:

The preparations were brought to water and then:

1. They were bathed in the fat stain in a closed container for 10 - 15 minutes.
2. The background staining was cleared by differentiating in 60% alcohol.
3. The preparations were washed in water and photographed.

e. Haematoxylin, phloxine and saffron - staining technique for methacrylate sections.

Staining solutions.

a)/

a) Lillies haematoxylin.

b) 1% Phloxine (aqueous).

c) Saffron (alcoholic).

The methacrylate embedded tissue sections were:

1. Rinsed in distilled water.
2. Stained in Lillies haematoxylin for 10 minutes.
3. Differentiated in 1% acid alcohol for 3 seconds.
4. 'Blued' in tap water for 10 minutes.
5. Stained in phloxine for 7 minutes.
6. Rinsed in water.
7. Dehydrated in two changes of alcohol.
8. Stained in saffron for 15 minutes.
9. Rinsed in two changes of xylene.
10. The preparations were then mounted in DPX.

4. SCANNING ELECTRON MICROSCOPY

i) Fixation.

Flasks which were to be examined with the scanning electron microscope were first washed with two changes of PBS at 37°C. The cells were then fixed with Cacodylate buffered glutaraldehyde and stored at 4°C until processed further.

ii) Post fixation.

The cells to be examined were washed first in cacodylate buffer and then several times in distilled water. They were then covered with a solution of 1% Osmium tetroxide in 0.2 M cacodylate buffer for 1 hour at room temperature. This procedure was carried out with extreme care in the fume cupboard as osmium tetroxide is a highly dangerous solution.

iii) Preparation of samples.

The cells were then washed several times with distilled water and the flasks filled to capacity with fresh distilled water. The area to be examined was cut out of the flask using a small 12 volt hand held electric drill fitted with a 13 mm circular saw (Expo). The pieces thus removed were labelled on their underside using a diamond marker, washed well in distilled water and the preparations were then progressively dehydrated using methanol and the following regime:

5 minutes each in:- 70%, 80%, 85%, 90%, 95%, 100%, 100%, methanol. The specimens were then placed in a Polaron critical point drier in methanol, and liquid CO_2 was flushed through the system until all traces of methanol were removed. The drying cycle was completed according to standard procedures as recommended by Bartlett and Burnstyn[12] in 1975.

Following/

Following drying the specimens were mounted on Cambridge ½" specimen stubs using Evostick 6503 electrically conductive adhesive.
The specimens were then coated with approximately 50 nm of 60% gold/40% Paladium in a Polaron E5000 sputter coater.

iv) Examination and Photography.

The specimens were examined in a Cambridge 600 scanning electron microscope and photographs were taken with a Practika L2 camera on Kodak Technical Pan film No. 2415.

5. TRANSMISSION ELECTRON MICROSCOPY

Cells were prepared and examined with the Transmission electron microscope in the Department of Pathology of Edinburgh University. The flasks containing the cells and medium were transported carefully to the University where the cells were fixed and processed for examination. The technique was essentially that outlined by Stuart et al [158] in 1978.

i) Fixation.

The medium was decanted from the flask and the cells were rinsed with 0.1 mol/l sodium cacodylate buffer.

The cells were fixed with 2 parts 1% osmium tetroxide in 0.1 mol/l sodium cacodylate buffer : 1 part 1.5% glutaraldehyde in 0.067 mol/l cacodylate buffer.

Fixation was carried out in an ice bath in the refrigerator for 10 - 15 minutes.

ii) Further preparation of samples.

Following fixation the cells were:

1. Rinsed in 0.1 mol/l sodium cacodylate buffer.
2. Rinsed twice in 70% alcohol. 5 minutes each change.
3. Treated to 3 x 15 minute changes of absolute alcohol.
4. 10 minutes treatment with 50:50 absolute alcohol:
   EPON (without accelerator).
5. 10 minutes treatment with 25:75 absolute alcohol:
   EPON (without accelerator).
6. 10 minutes treatment with 5:95 absolute alcohol:
   EPON (without accelerator).
7. 2 X 10 minutes 100% EPON (without accelerator).
8. 1 X 10 minutes 100% EPON with accelerator.

9./

9. A hole was then cut in the top of the flask to ensure that there was even polymerisation, due to removal of the excess alcohol, and the flask was incubated at 37°C for 2 - 3 weeks.
10. Excess plastic was removed from the flask and one end of the flask was cut so that there was an EPON/plastic join visible.
11. The flask was then placed in boiling water, to soften the EPON slightly, and the EPON/cell layer was peeled off the flask plastic.
12. The EPON was cut into blocks and these were stuck onto Araldite blocks using Araldite.
13. A pyramid was cut and thin sections were cut using glass knives.
14. The sections were picked up onto 200 mesh uncoated copper grids (Athens).
15. The sections were stained with uranyl acetate and lead citrate.

iii) Examination and photography.

The specimens were examined with an AEI Corinth 275 electron microscope and photographs were taken with a 70 mm Camera on Ilford IC4 film.

RESULTS

RESULTS

INTRODUCTION

The original purpose of the work for this thesis was (pp 2) to grow and observe, in culture, cells from highly elastotic breast tumours, as a possible means of gaining an insight into cancers which have been shown to be associated with a relatively good prognosis.[105, 150, 172]

The proportion of all breast cancers which may be defined as "highly elastotic" has been put, in three reported studies, at 17% of scirrhous cancers[8] and 23% and 16% of all types of breast cancer.[150]

Over a period of 34 months mammary tissue from 74 patients was set up in culture and the growth characteristics of the cells which survived and grew were studied. In the present series, owing to a certain degree of selection, 27% (15/55) of the neoplastic tissues cultured were assigned an "elastica index" of 11,[150] that is, lesions containing thick and dense aggregates of elastica so numerous as to dominate the histological picture.

The tissue cultured was taken from:

1 "Normal" breast.

1 Breast at 18 weeks gestation.

17 Non-malignant breast lesions.

1 Cystosarcoma phyllodes.

39 Carcinomas - Elastosis Grade 1 and 0.

15 Highly elastotic breast tumours (Grade 11)

Many accounts of the cultural characteristics of breast tissue have appeared in the literature; few, however, have proceeded from the basic premise that some or all of the connective tissue of a tumour is tumour-cell derived.

During/

During the course of the investigation a large variety of both "epithelial-like" and "fibroblast-like" cells was recognised. Their differences, often subtle and inconstant, are, in the present state of knowledge, almost beyond any but the most tentative interpretation. In order not to obscure the findings central to the present thesis, descriptions of the various cell types, together with their incidence and representative photographic evidence, are contained in Section VI (p.191).

NOTE

Unless stated otherwise, all photomicrographs were taken at an original magnification of 150.

Fig. 1.

TC 67. Lobular carcinoma with a high degree of elastosis. 7 days culture.

Low power view of elastotic cells showing 'blebbing' phenomenon. Phase contrast.

Original magnification x 150

SECTION 1.

THE CULTURAL CHARACTERISTICS OF HIGHLY ELASTOTIC BREAST TUMOURS.

i) Light Microscopy

Fifteen highly elastotic breast tumours were grown in culture and, as with all the other tissue cultured, a variety of morphological cell types grew. The incidence of each cell type, together with a brief description, is contained in Section VI. (p. 191).

One cell type, here designated the "Elastotic cell" appeared almost exclusively in cultures of cells from those tumours which contained elastica.

ELASTOTIC CELLS

Elastotic cells varied in their outline from rather elongated and fusiform to a polygonal shape. The feature which separated these cells from all others cultured was the persistent "blebbing" of material from the surface of the cell. This phenomenon was marked, under phase contrast microscopy, by the presence of both refractile and greyish excrescences which were extruded from the cell into the medium. Release of the "blebbed" material often appeared to lead to the production of a vacuole within the cell. Occasionally entire cells detached from the flask floor and were seen floating as shiny balls in the medium.

TC 67. Lobular carcinoma. High degree of elastosis. 7 days.

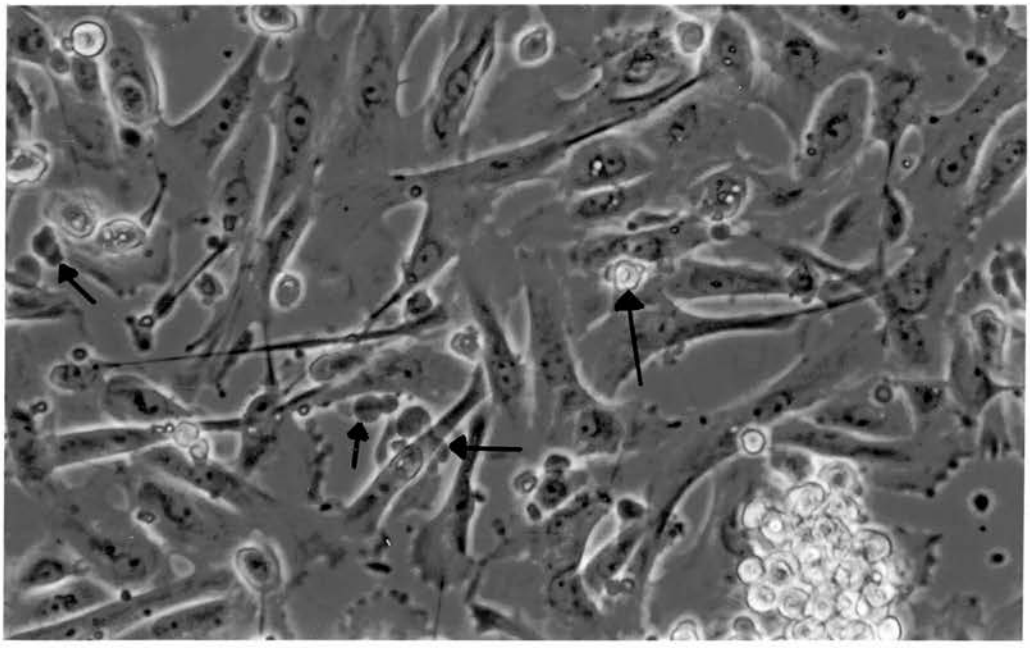


Fig. 2.

TC 67. Lobular carcinoma with a high degree of elastosis. 6 days culture.

Low power view of elastotic cells showing 'blebbing' phenomenon. Phase contrast.

Original magnification x 150

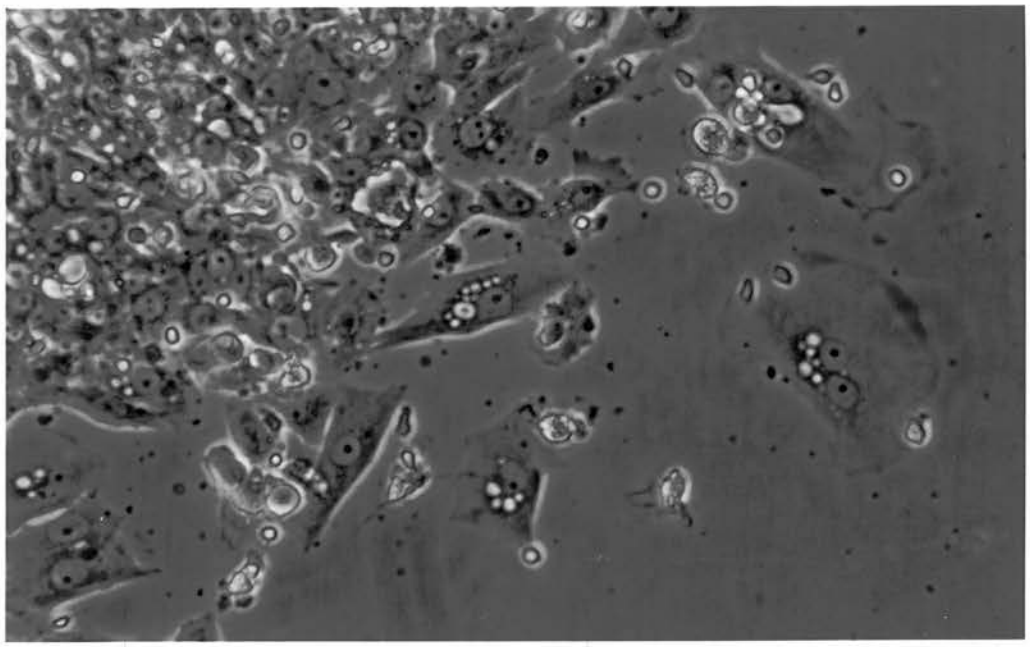
Fig. 3.

TC 67. Lobular carcinoma with a high degree of elastosis. 7 days culture.

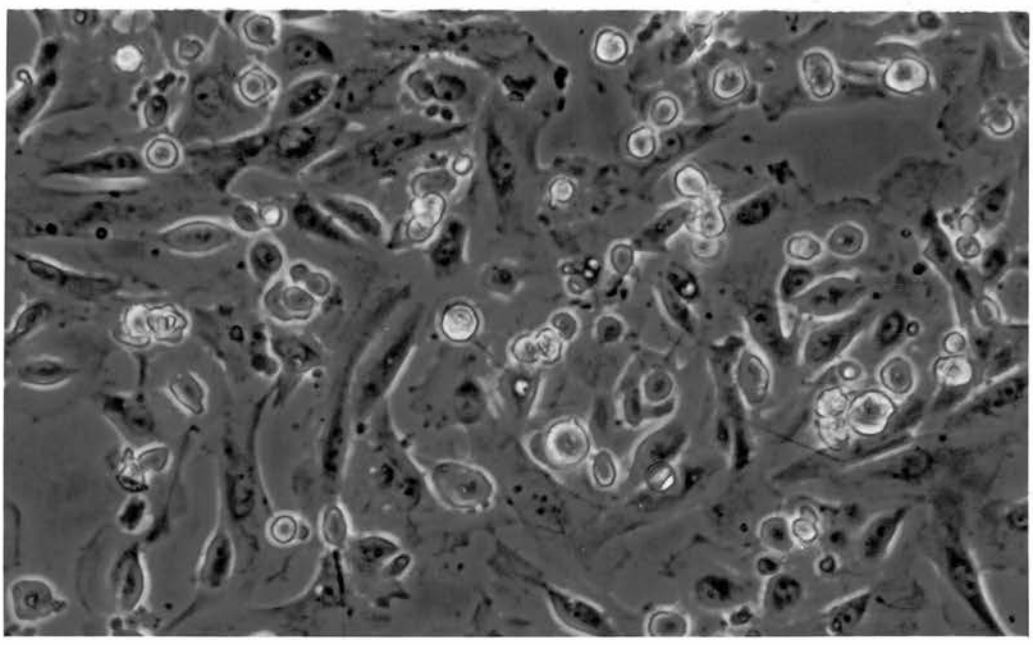
Low power view of elastotic cells showing 'blebbing' phenomenon. Phase contrast.

Original magnification x 150

TC 67. 6 days.



TC 67. 7 days.



Figs. 4 and 5.

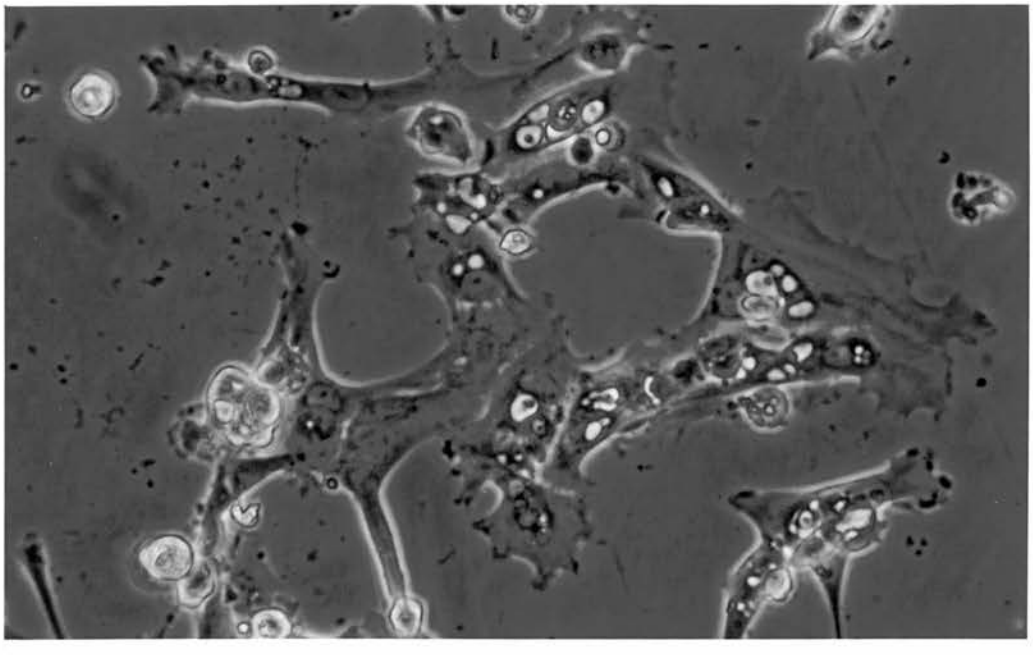
TC 67. Lobular carcinoma with a high degree of elastosis. 8 days culture.

Low power views of elastotic cells showing blebs and vacuoles. Phase contrast.

Original magnification x 150

TC 67.

8 days.



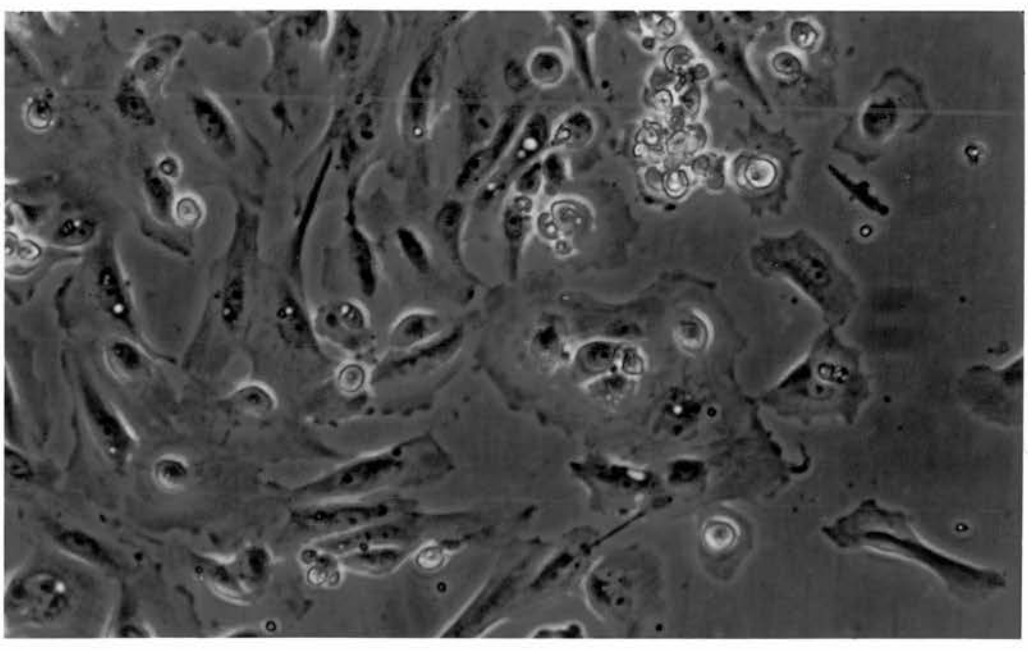


Fig. 6.

TC 77. Anaplastic carcinoma. Grade I elastosis. 6 days culture.

Low power view of elastotic cells showing blebs ⟵ and vacuoles.

Phase contrast. Original magnification x 150

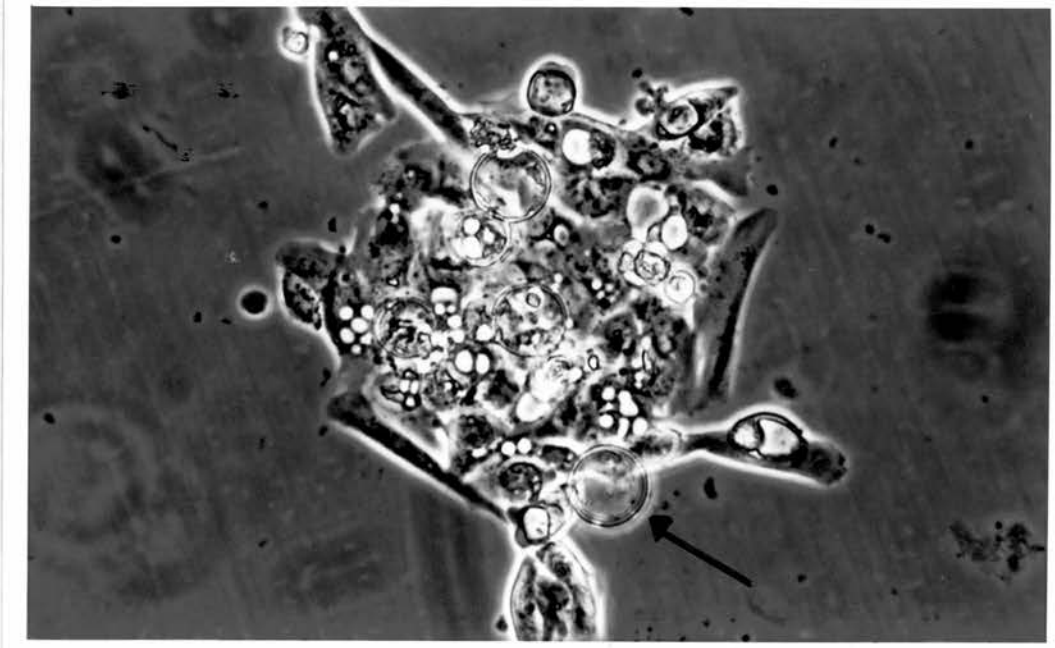
Fig. 7.

TC 77. Anaplastic carcinoma. Grade I elastosis. 6 days culture

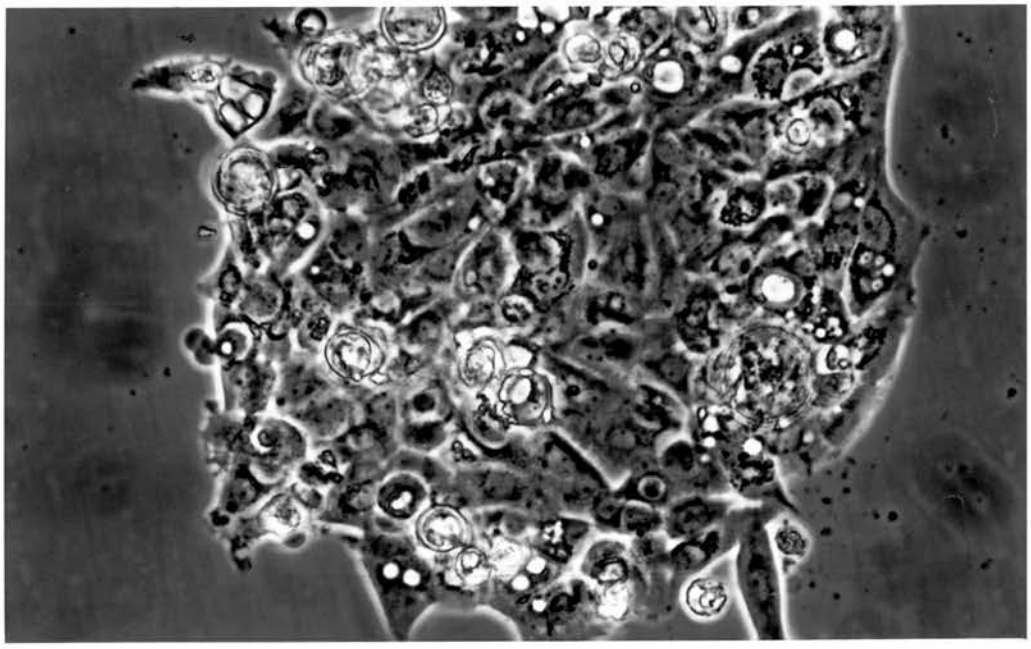
Low power view of elastotic cells. Phase contrast.

Original magnification x 150.

TC 77. Anaplastic carcinoma. Grade I elastosis. 6 days culture.



TC 77. 8 days.



Figs. 8 and 9.

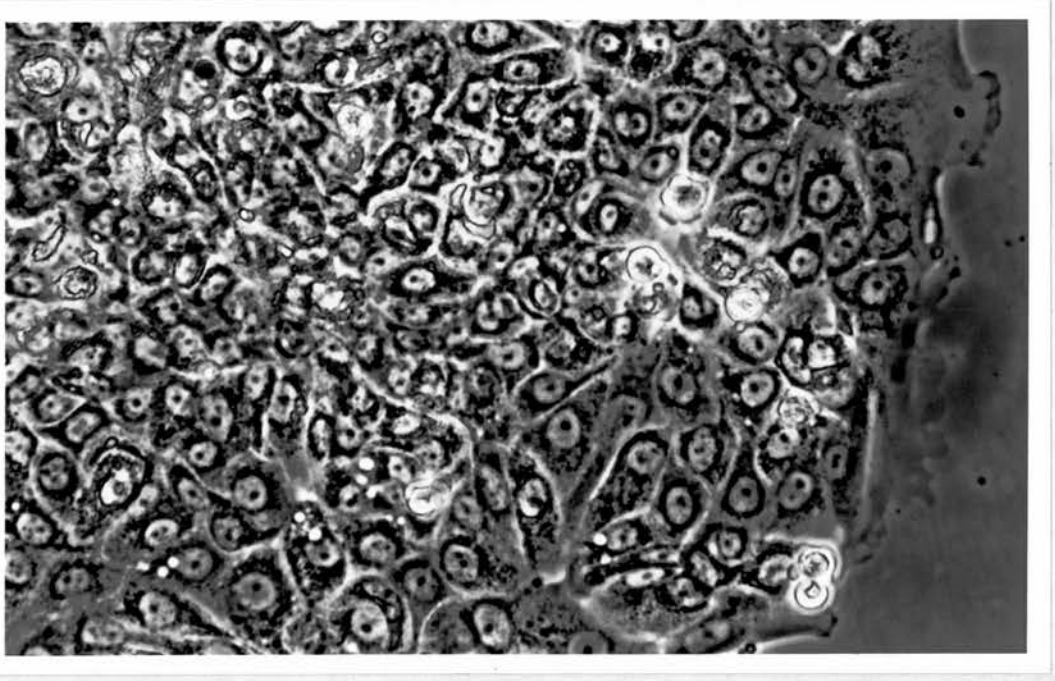
TC 76. Anaplastic carcinoma. Grade II elastosis. 6 days culture.

Low power view of elastotic cells. Phase contrast.

Original magnification x 150

Note blebs, vacuoles and granular cytoplasm in TC 76 cells.

TC 76. Anaplastic carcinoma. Grade II elastosis. 6 days culture.



TC 76. 6 days.

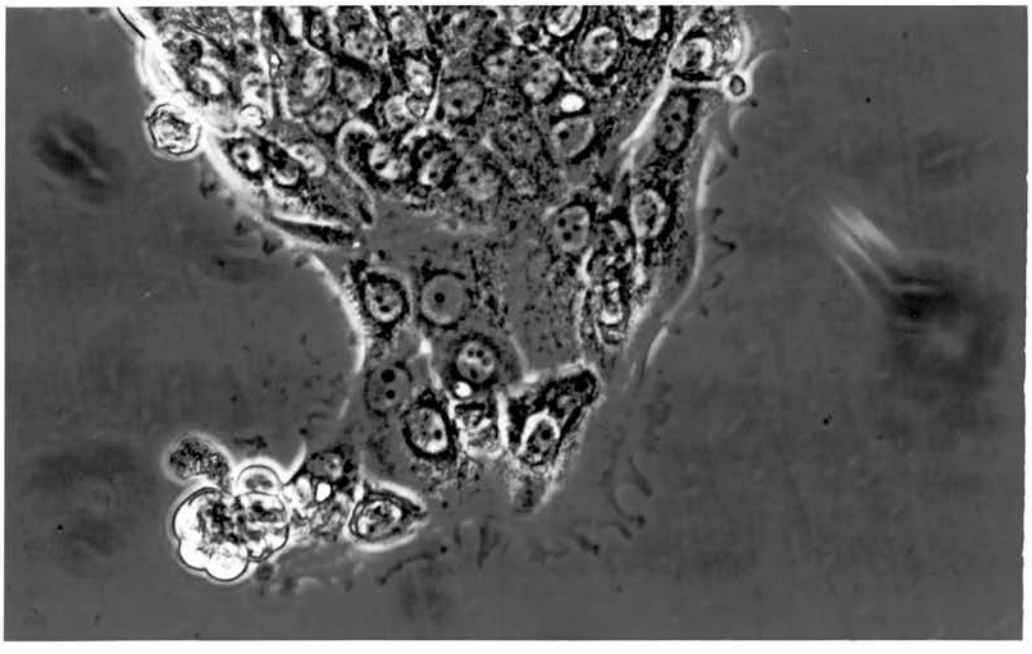


Fig. 11.

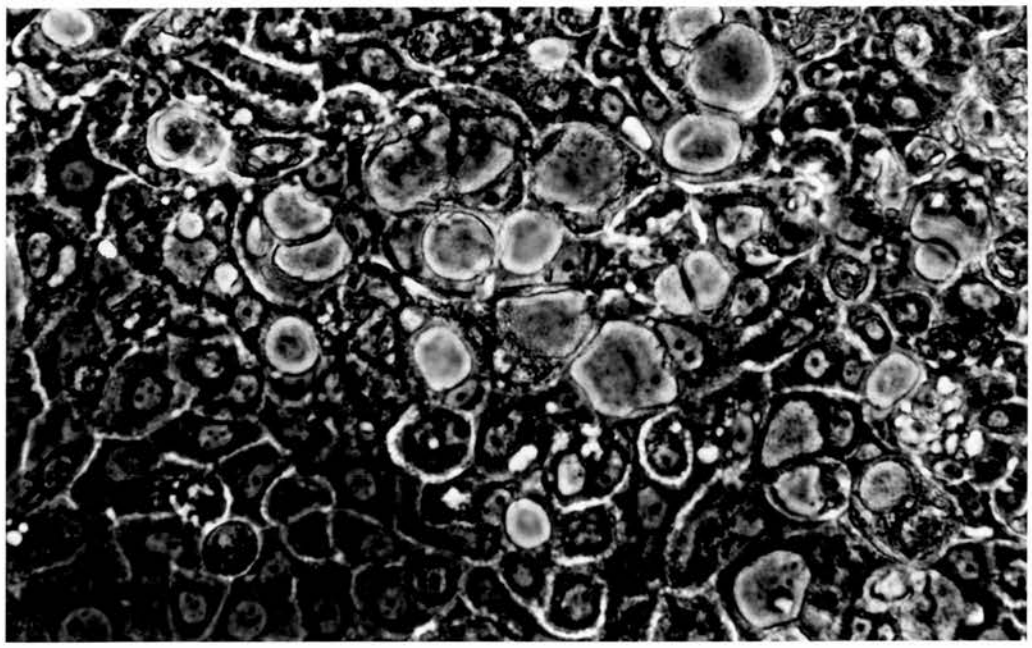
TC 80. Anaplastic carcinoma. Grade II elastosis. 6 days culture.

Low power view of elastotic cells. Phase contrast.

Original magnification x 150

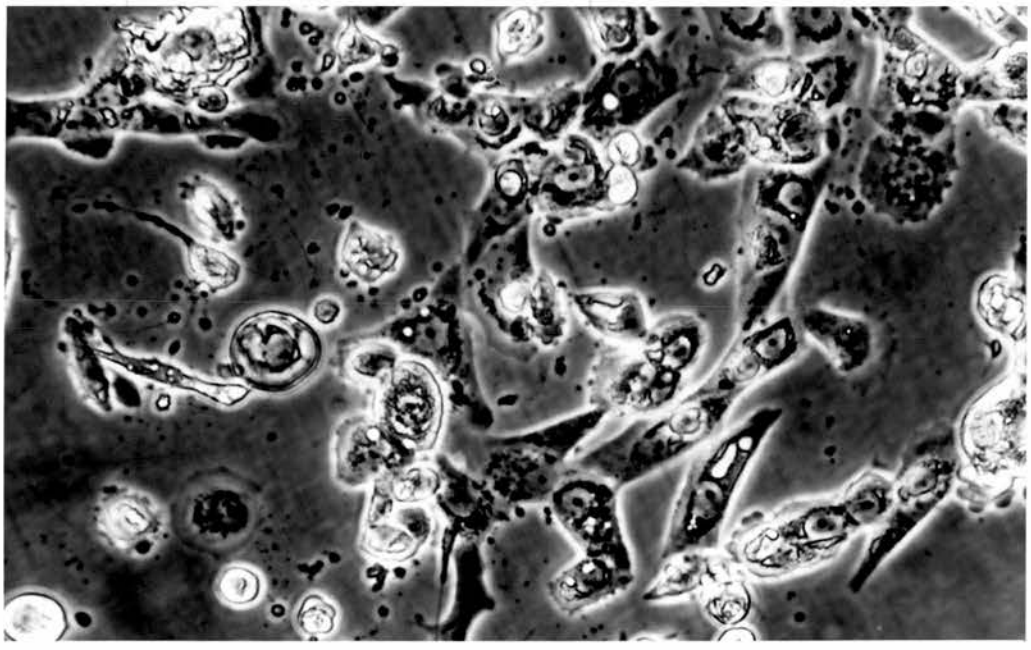
Fig. 10.

TC 76. Anaplastic carcinoma. Grade II elastosis. 10 days.



The appearance of the cells, here designated "elastotic cells" is the same as those identified as "apocrine cells" in Section III.

TC 80. Anaplastic carcinoma. Grade II elastosis. 6 days.



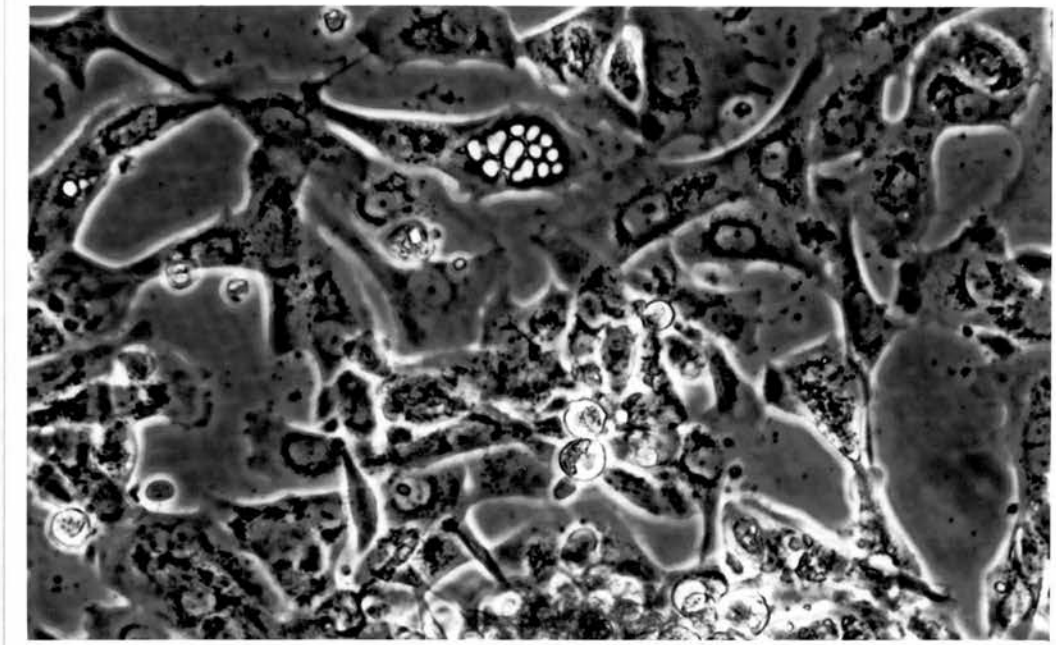
Figs. 12 and 13.

TC 80. Anaplastic carcinoma. Grade II elastosis. 8 days culture.

Low power views of elastotic cells showing blebs and vacuoles.

Phase contrast. Original magnification x 150

TC 80. Anaplastic carcinoma. Grade II elastosis. 8 days.



TC 80. 8 days.

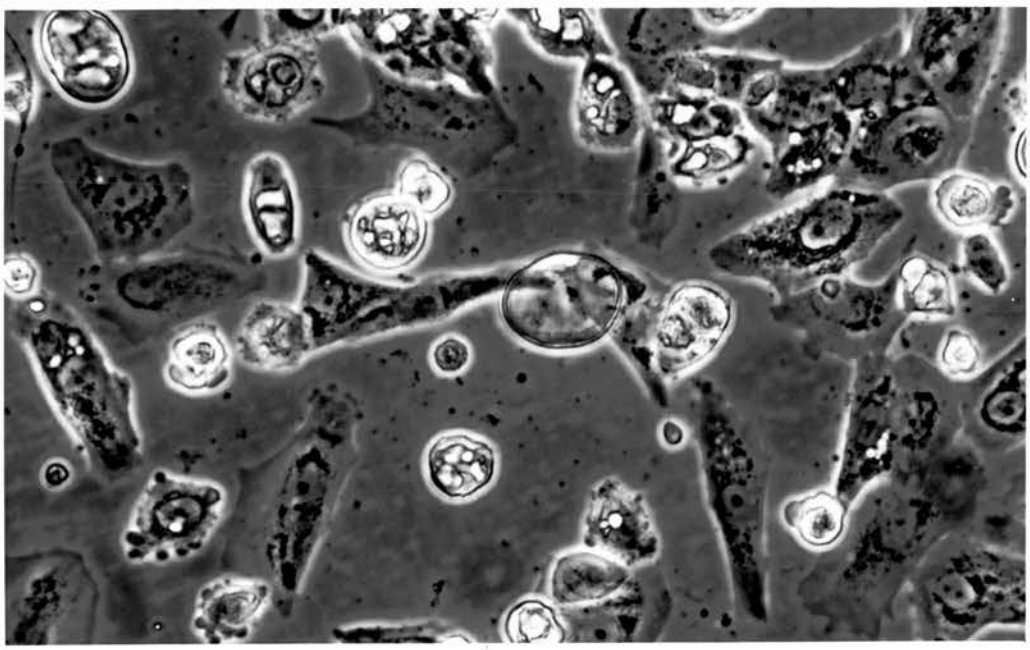


Fig. 14.

TC 82. Anaplastic carcinoma. Grade II elastosis. 8 days culture.

Low power view of polygonal elastotic cells showing blebs. Phase contrast.

Original magnification x 150

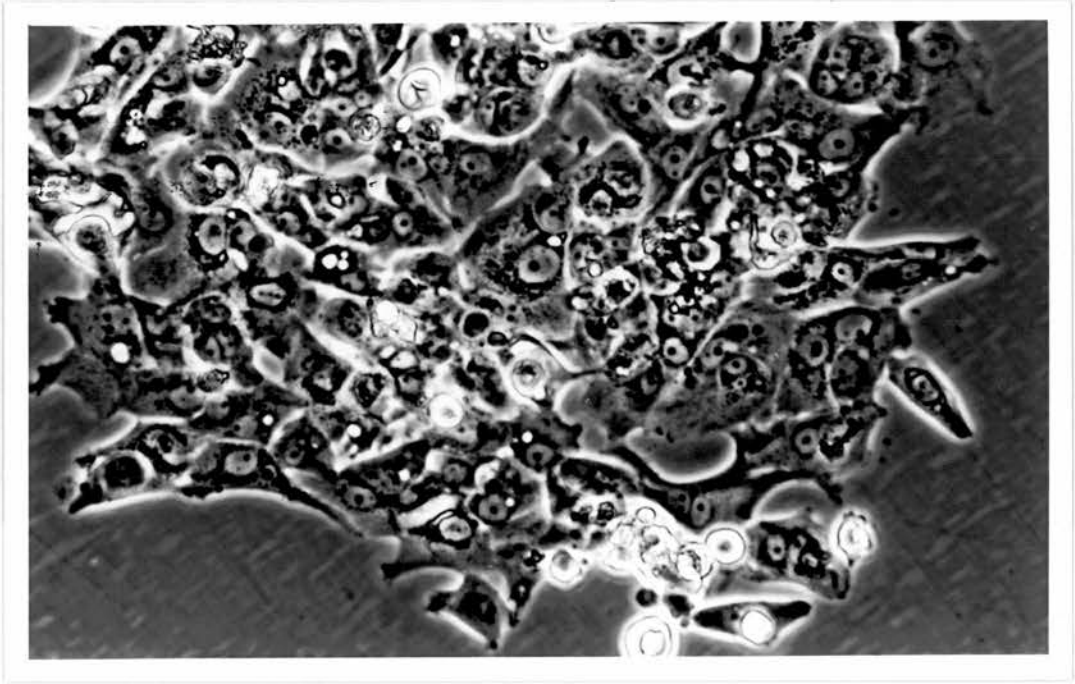
Fig. 15.

TC 82. Anaplastic carcinoma. Grade II elastosis. 13 days culture.

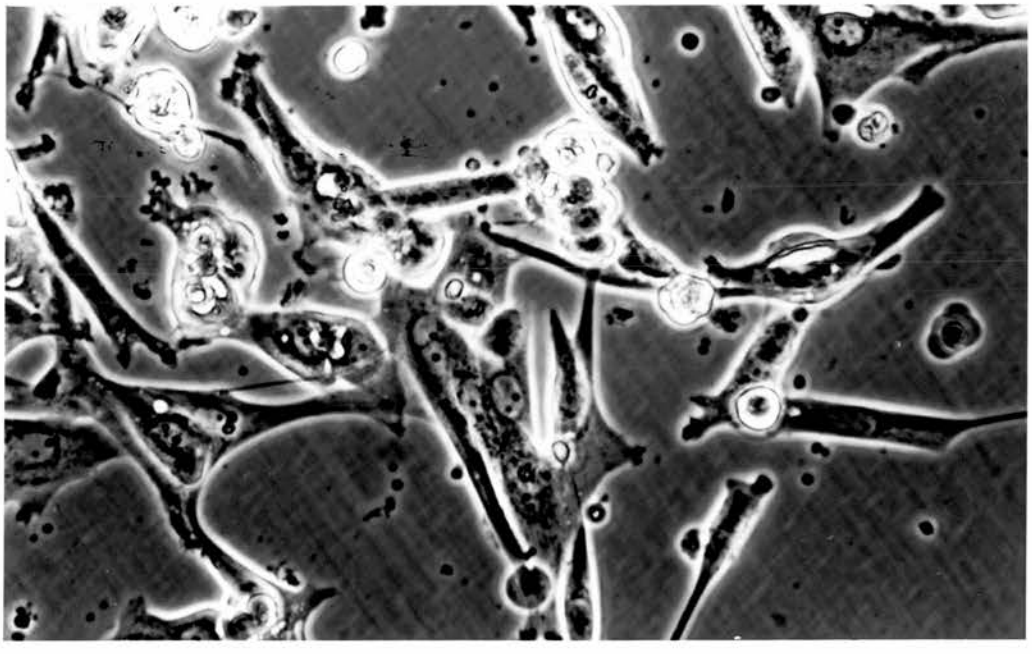
Low power view of elongated elastotic cells showing blebs. Phase contrast.

Original magnification x 150

TC 82. Anaplastic carcinoma. Grade II elastosis. 8 days.



TC 82. 13 days.



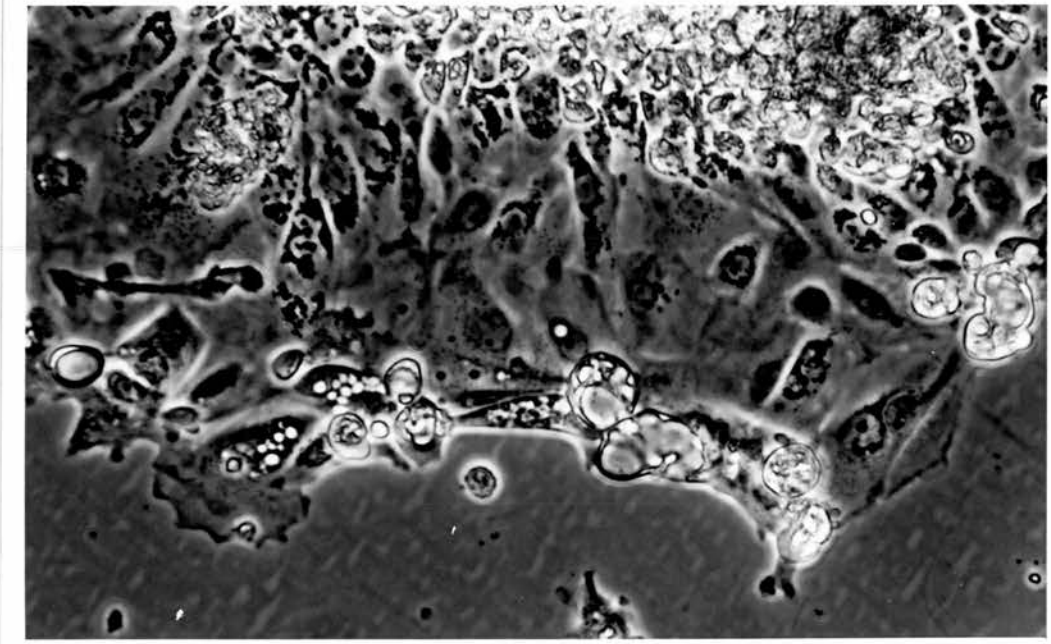
Figs. 16 and 17.

TC 83. Anaplastic carcinoma. Grade II elastosis. 7 days culture.

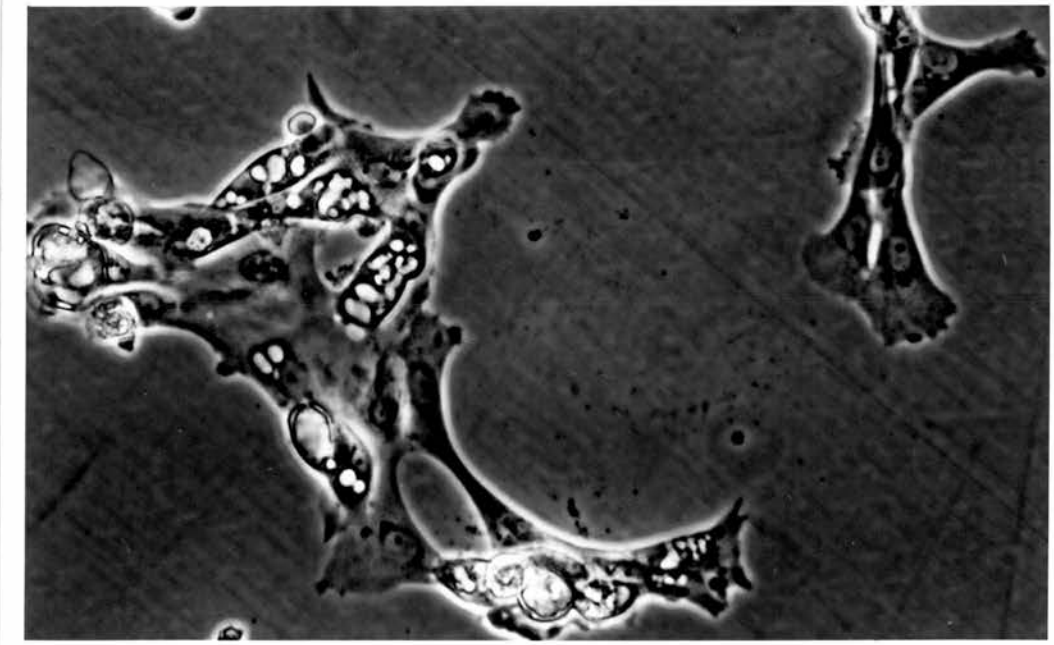
Low power view of elastotic cells. Phase contrast.

Original magnification x 150

TC 83. Anaplastic carcinoma. Grade II elastosis. 7 days culture.



TC 83. 7 days.



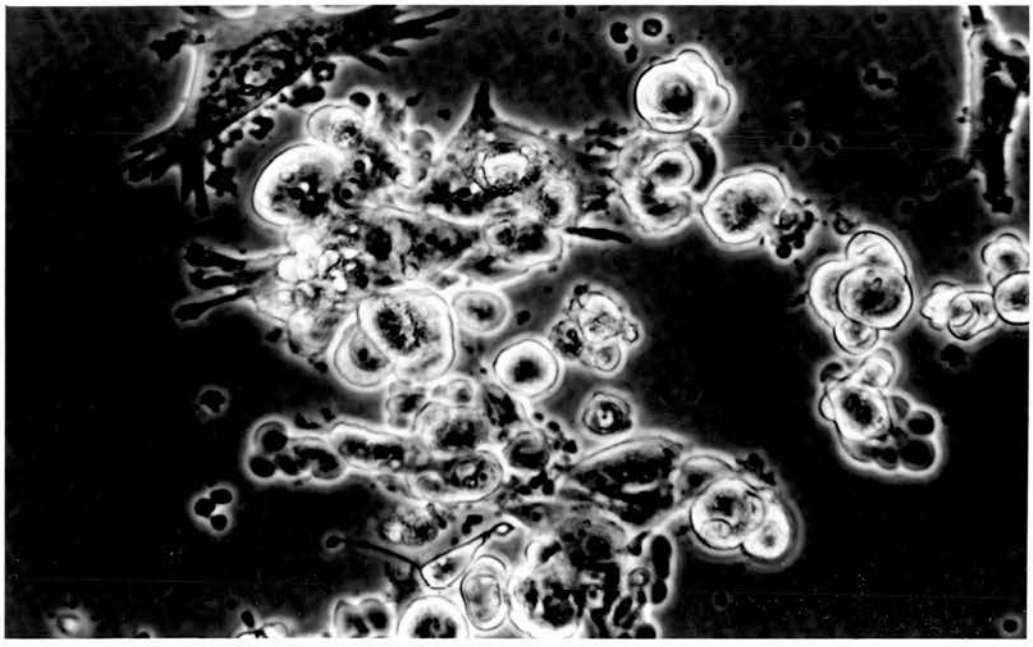
Figs. 18 and 19.

TC 86. Anaplastic carcinoma. Grade II elastosis. 6 days culture.

Low power view of elastotic cells showing marked blebbing. Phase contrast.

Original magnification x 150

TC 86. Anaplastic carcinoma. Grade II elastosis. 6 days culture.



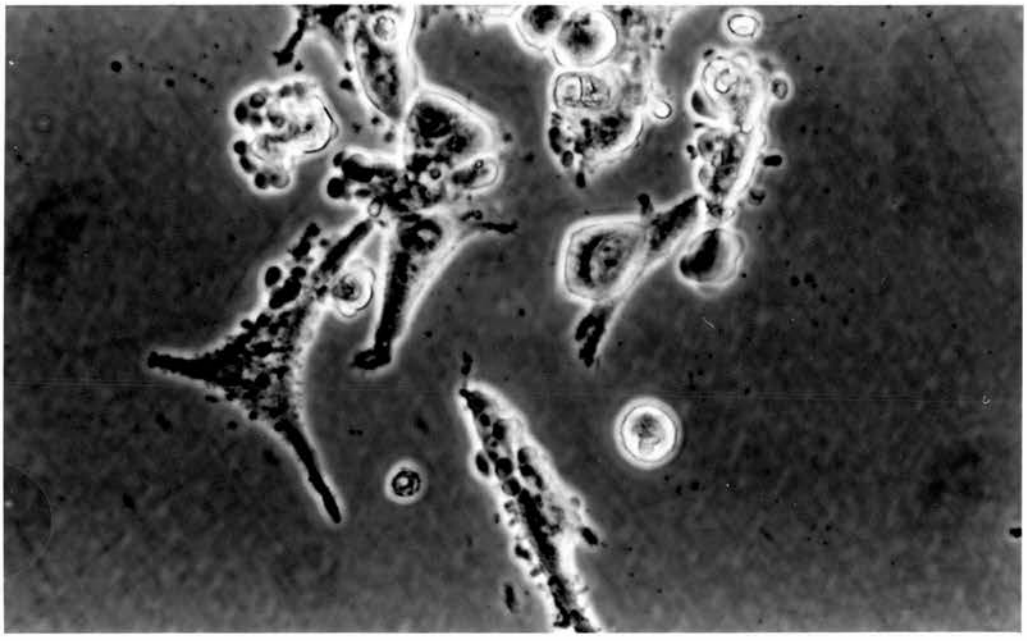


Fig. 20.

TC 30. Anaplastic carcinoma. Moderate elastosis. 9 days culture.

Low power view of elastotic cells showing marked blebbing. Phase contrast.

Original magnification x 150

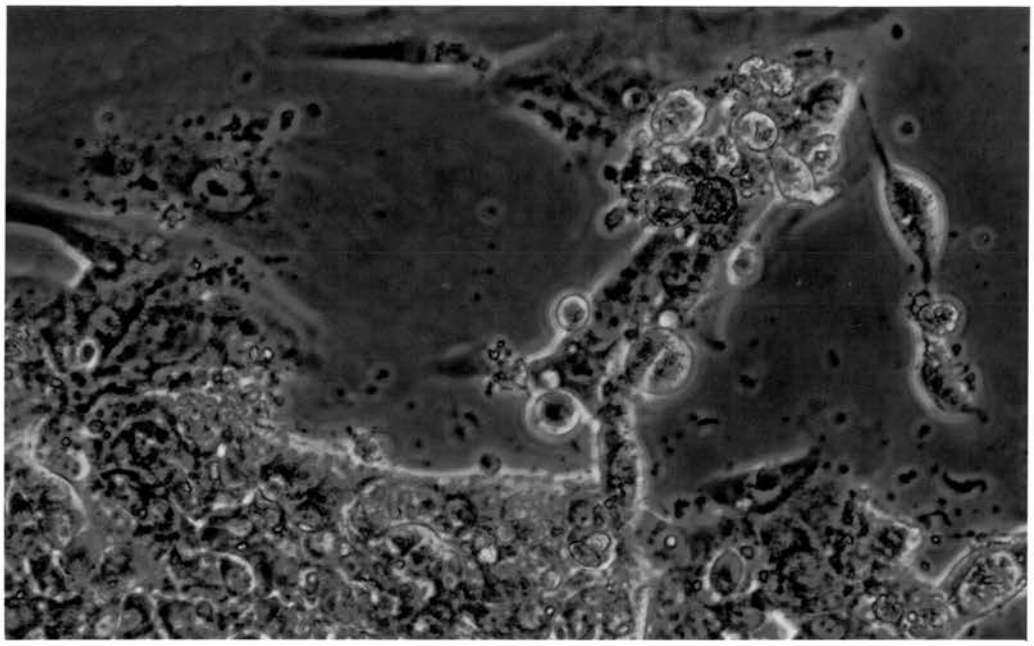
Fig. 21.

TC 30. Anaplastic carcinoma. Moderate elastosis. 37 days culture.

Low power view of elastotic cells showing marked blebbing. Phase contrast.

Original magnification x 150

TC 30. Anaplastic carcinoma. Moderate elastosis. 9 days culture.



TC 30. 37 days.

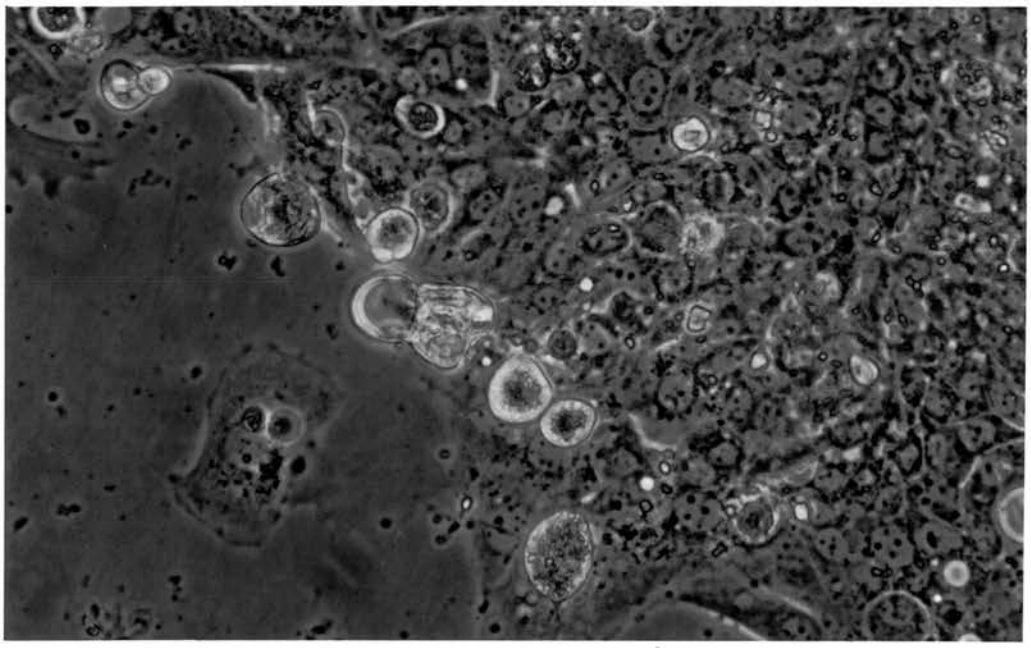


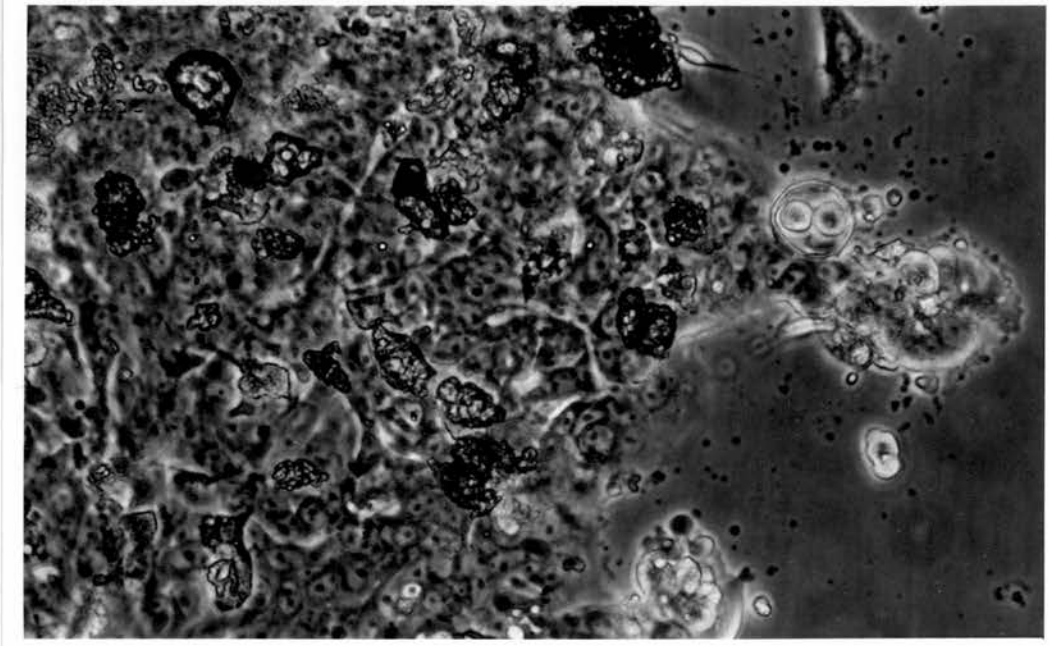
Fig. 22.

TC 33. Anaplastic small cell carcinoma. Moderate stromal reaction; moderate elastosis. 6 days culture.

Low power view showing blebs and extruded material. Phase contrast.

Original magnification x 150

TC 33. Anaplastic small cell carcinoma. Moderate stromal reaction, moderate elastosis. 6 days culture.



Figs. 23 and 24.

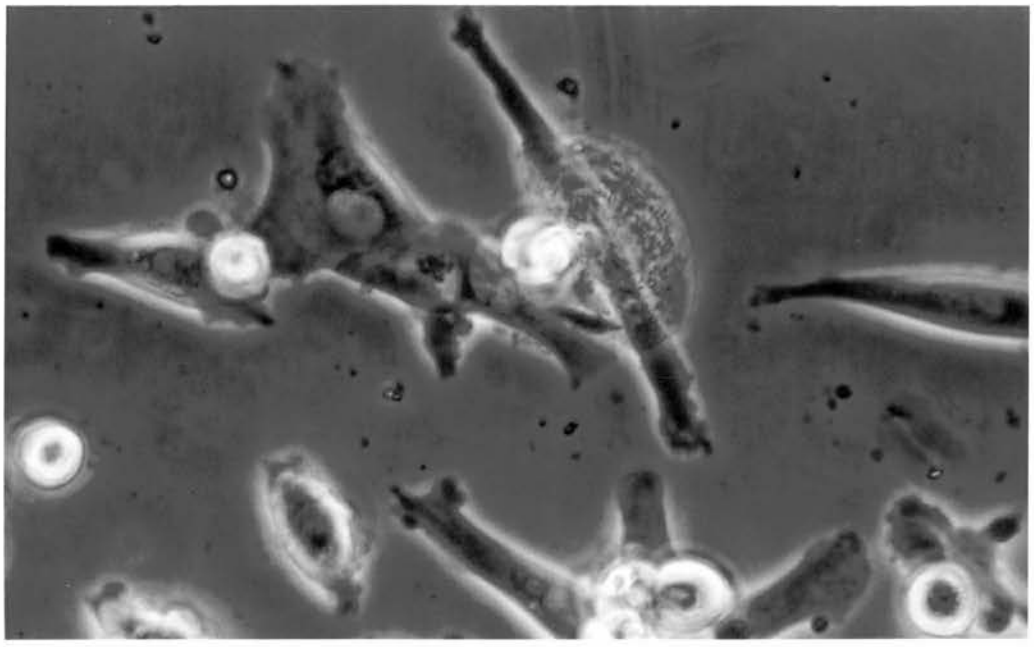
TC 66. Anaplastic carcinoma. Grade II elastosis. 21 days culture.

Detailed views of material being actively extruded from elastotic cells.

Phase contrast. Original magnification x 300

At a higher magnification (original magnification X 300), the phenomenon is illustrated more clearly:-

TC 66. Anaplastic carcinoma. Grade II elastosis.



TC 66.

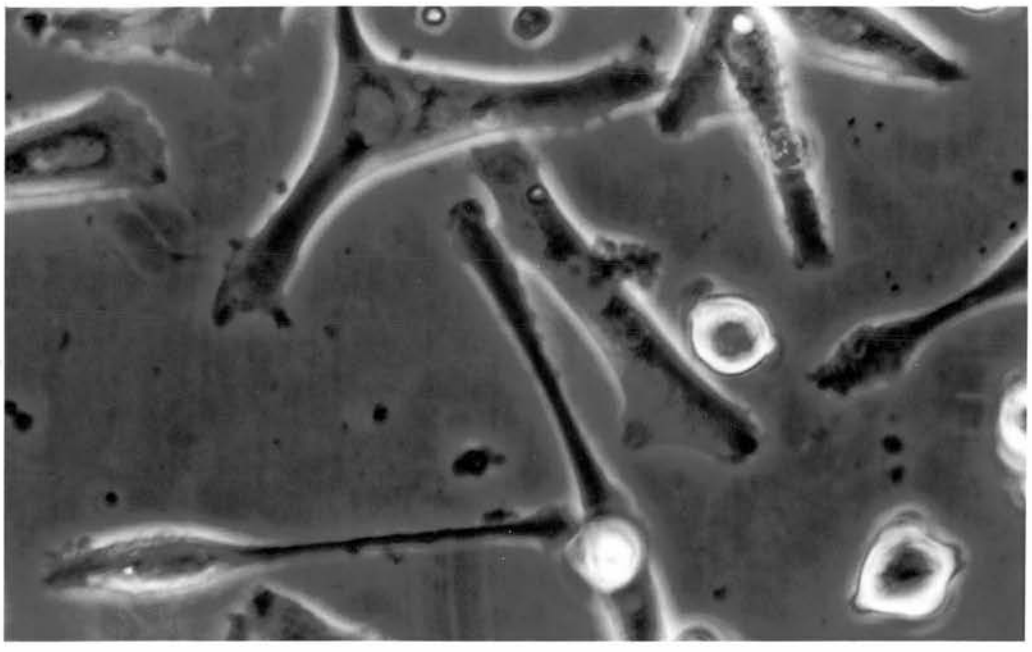


Fig. 25.

TC 67. Lobular carcinoma with a high degree of elastosis. 7 days culture.

Detailed view of 'blebbing' phenomenon. Phase contrast.

Original magnification x 300

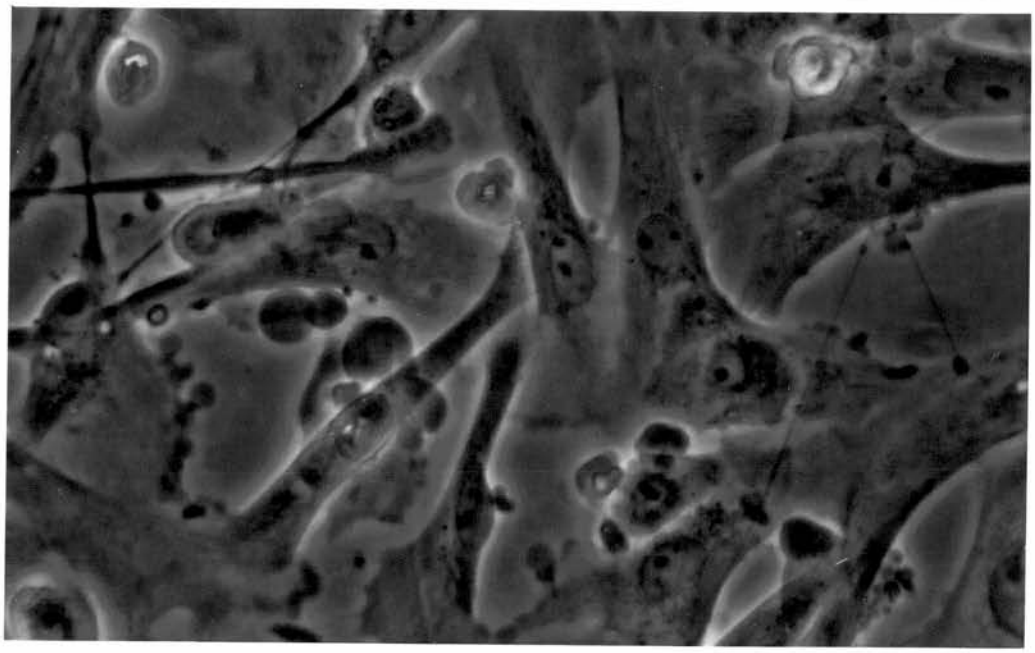
Fig. 26.

TC 76. Anaplastic carcinoma. Grade II elastosis. 22 days culture.

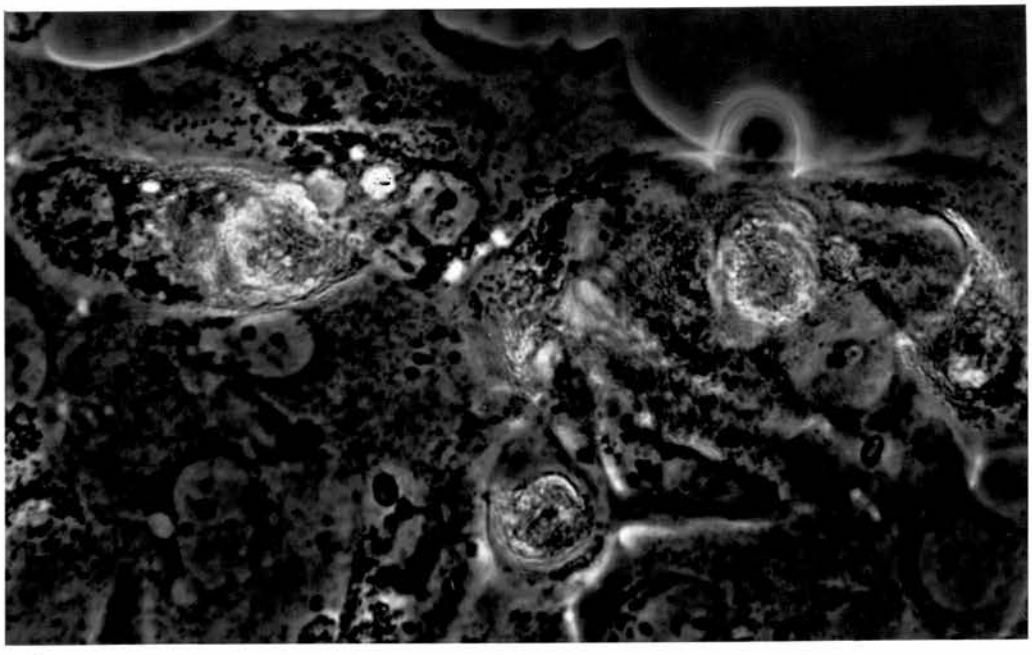
Detailed view of granular elastotic cells showing blebbing. Phase contrast.

Original magnification x 300

TC 67.



TC 76.



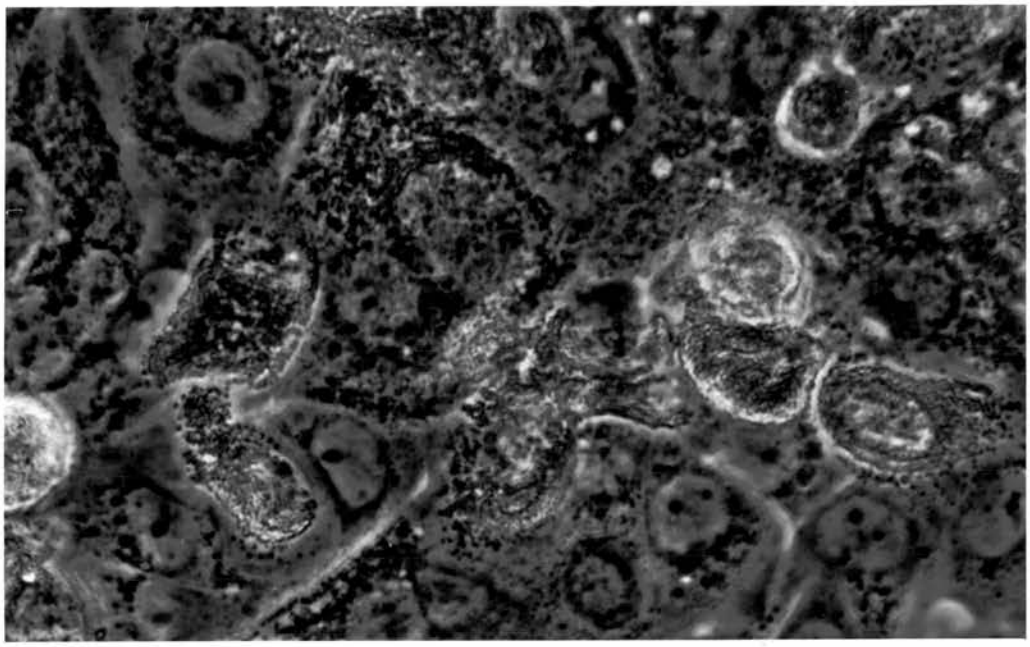
Figs. 27 and 28.

TC 76. Anaplastic carcinoma. Grade II elastosis. 22 days culture.

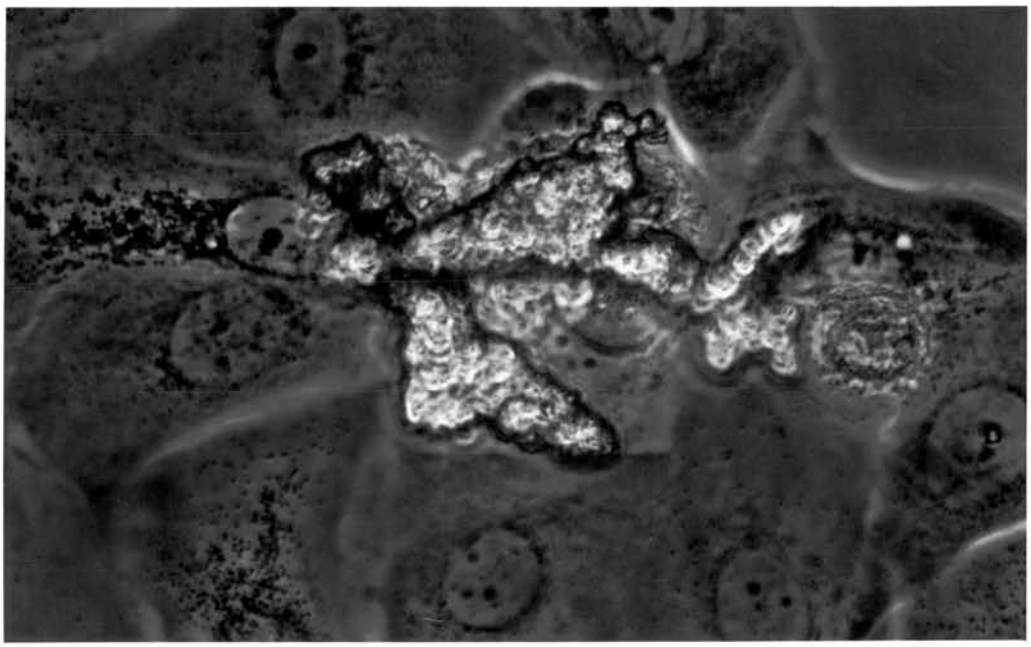
Detailed view of granular elastotic cells showing blebs and excrescences.

Phase contrast. Original magnification x 300

TC 76.



TC 76.



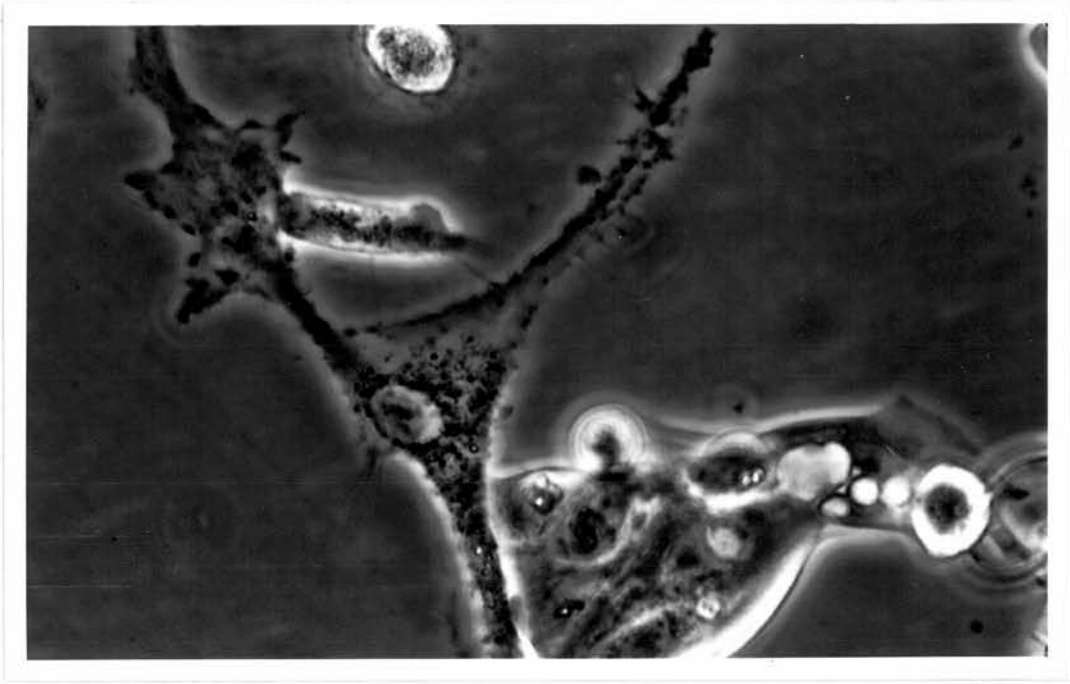
Figs. 29 and 30.

TC 82. Anaplastic carcinoma. Grade II elastosis. 14 days culture.

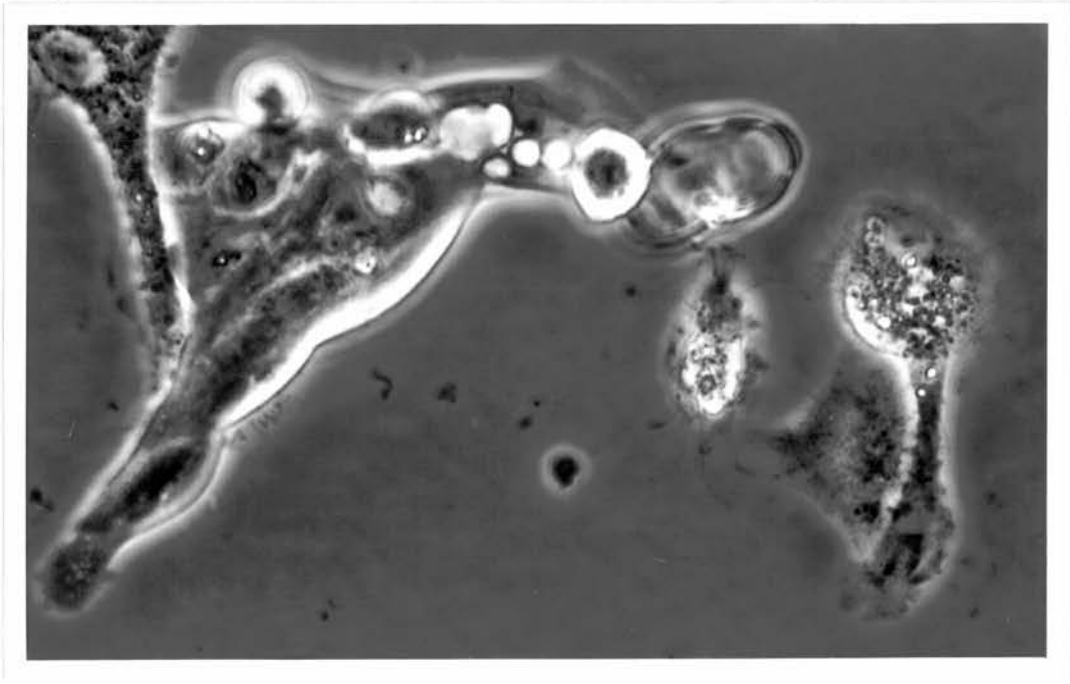
Detailed views of blebbing phenomenon. Phase contrast.

Original magnification x 300

TC 82.



TC 82.



Cells which markedly resembled those illustrated were observed in eight of the fifteen highly elastotic tumour cell cultures. A lesser degree of resemblance was noted in a further four. "Elastotic cells" were also seen in two out of seven slightly elastotic tumour cultures and a further two cultures contained cells which bore a slight resemblance to these cells. (See Table IV). There were also cells which were similar to "elastotic cells" in five of the cultures from tumours in which no elastica was noted at diagnosis. It is possible that these cells were, indeed, elastotic cells as few breast tumours (10 - 12%) are completely devoid of elastica.[8, 150]

Elastotic cells did not extrude material continuously but appeared to "bleb" actively for several days and then pass into a "resting" phase. The discontinuous nature of the phenomenon may be a reflection of the phasic deposition of elastica which is thought to occur in breast tumours (A.A. Shivas, personal communication).

It may be that "elastotic cells" were not identified in all highly elastotic tumour cultures because the cells were not, at the time of collection, in an active "producing phase". Alternatively it is possible that cells of that type were not present in the piece of tissue cultured, or that "elastotic cells" failed to survive and grow.

Exhaustive search of the literature for photographs which might depict the "elastotic cell", as cultured by other workers, was largely unsuccessful. One possible photomicrograph appeared in a paper by Hallowes et al[73] under the title of E^1 cells, cells which were found by them, as here, only in cultures of neoplastic tissue. These authors mention in their paper that E^1 cells:

> "frequently gave rise to domes, as well as to elongated tubes which rose several millimetres above the dish surface. These structures sometimes detached giving rise to spheres (of cells)."

In/

In the same paper Hallowes et al mention that raised blebs or domes appeared irregularly in cultures of E cells, cells which grew from lacteal secretions, benign mammary tissues and primary carcinomas. It is possible, therefore, that other workers have cultured "elastotic cells". Their association with the highly elastotic tumour has not, however, been recognised.

ii) Scanning Electron Microscopy

In order to gain a three-dimensional view of the "blebbing" phenomenon, observed using phase contrast microscopy, cultures of "elastotic cells" were examined with the scanning electron microscope.

This method of examination illustrated the phenomenon very graphically and revealed excrescences of many different shapes and sizes emerging from the surfaces of the cells. The occurrence of "blebs" on the surface of cells has been noted before by other workers using the scanning electron microscope [5, 65, 131, 132, 133, 134, 143, 167, 168, 171] and, indeed, blebbing is characteristic of the G_1 phase of the cell cycle [132], that is, the period which leads up to and prepares for DNA replication (the S period). The size of the blebs reported by these workers was, however, rarely above 4 μm in diameter [133] and generally approximately 2 μm [5]. The excrescences which occurred on "elastotic cells" were usually between 5 μm and 10 μm in diameter.

The surface structure of the blebs, as of the cells from which they protruded, was very varied. Some were comparatively smooth, some had short, stubby microvilli on their surfaces, 0.2 - 0.4 μm in diameter, others had blebs covered in ruffles. Many of the cells had long filopodia projecting many microns from the cell of origin. Some cells possessed both small blebs and microvilli. There was also evidence in some of the cultures of the clumped microvilli taken by Spring-Mills and Elias [155] to be indicative of a malignant or premalignant tissue. Occasionally "craters", approximately 5 μm in diameter, were visible in the cytoplasm. It is possible that these craters were caused by release of an elastotic bleb into the medium. See Fig. 47.

The/

The following micrographs are representative of the type of surface features observed in cultures from highly elastotic breast tumours.

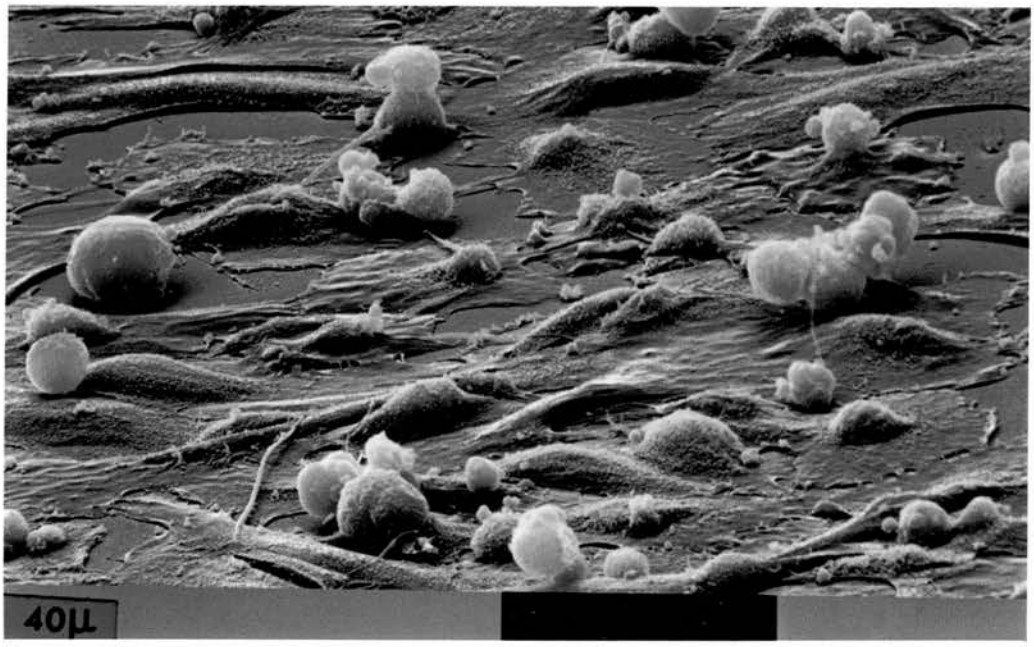
The degree of magnification is indicated by the black bar at the bottom of the photomicrograph. In each case the black bar represents the number of microns depicted in the left hand corner of the photograph.

Figs. 31 and 32.

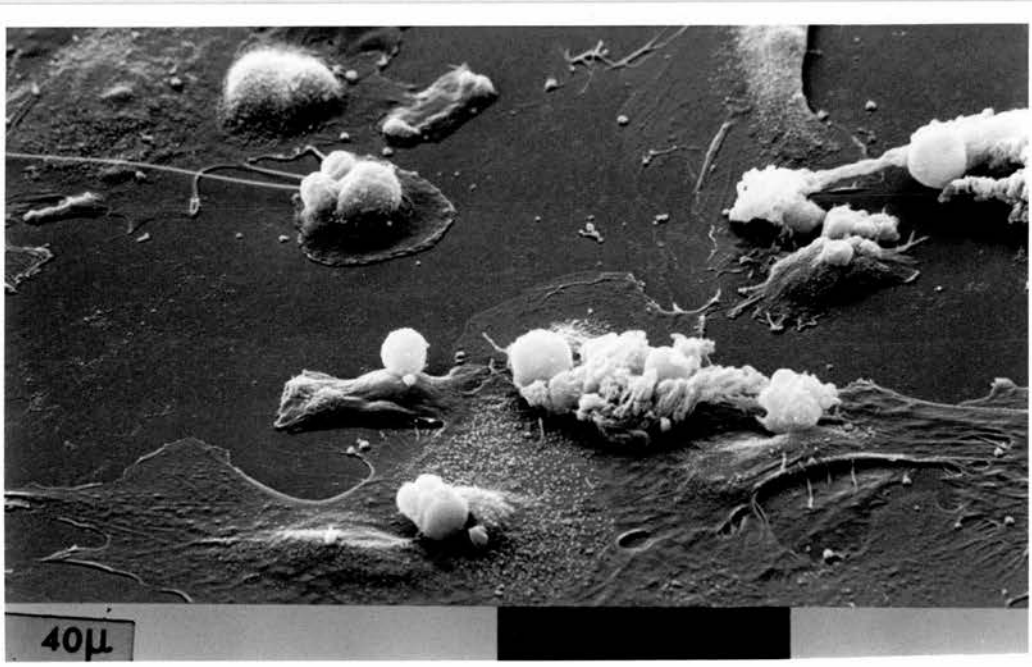
TC 67. Lobular carcinoma with a high degree of elastosis. 7 days culture. General view of elastotic cells growing on the floor of the culture flask showing cytoplasmic excrescences of various shapes and sizes emerging from the cell surfaces.

These cells are similar to those depicted in Figs. 1 - 5 (pp. 48 - 50).

TC 67. 7 days culture showing cytoplasmic excrescences of various shapes and sizes emerging from the cell surface.



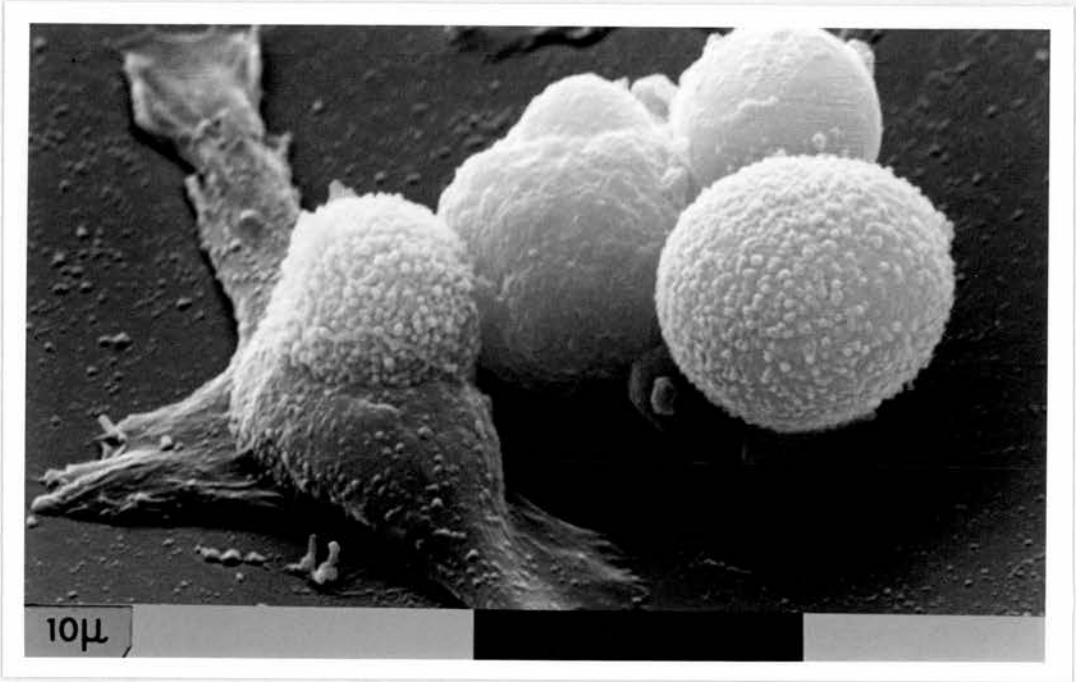
TC 67.



Figs. 33 and 34.

TC 67. 7 days culture. Detailed views of cytoplasmic excrescences illustrating the variation in the surface features.

TC 67. 7 days.



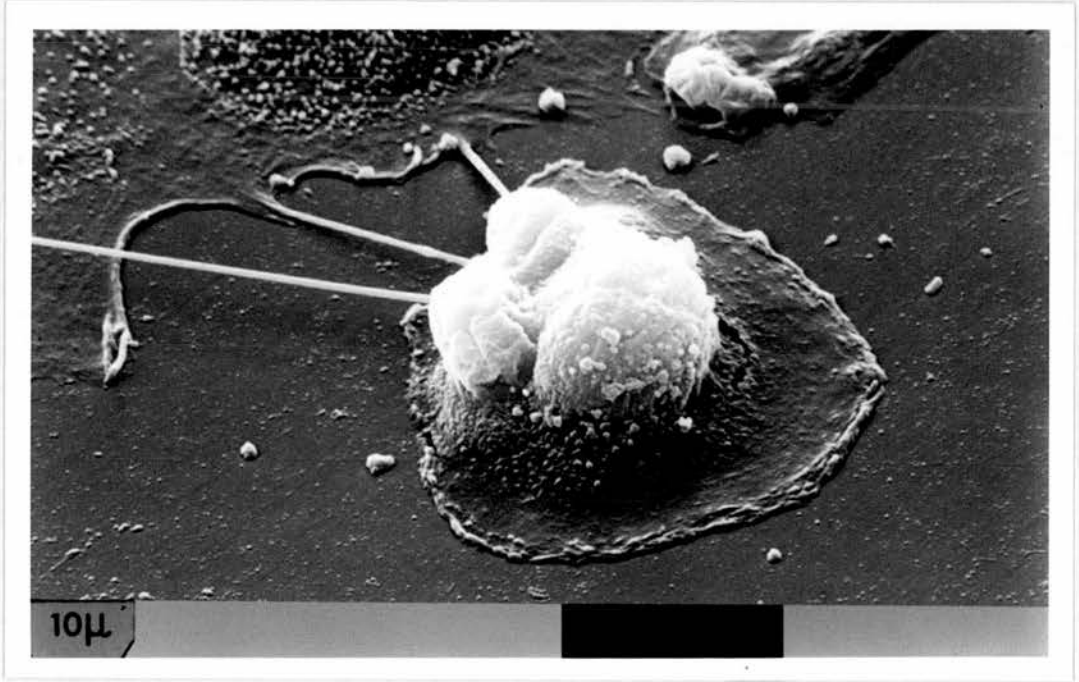


Fig. 35.

TC 67. 7 days culture. A large excrescence, approximately 10 µm in diameter, emerging from a well spread cell. Note the continuity between the cell and the "bleb".

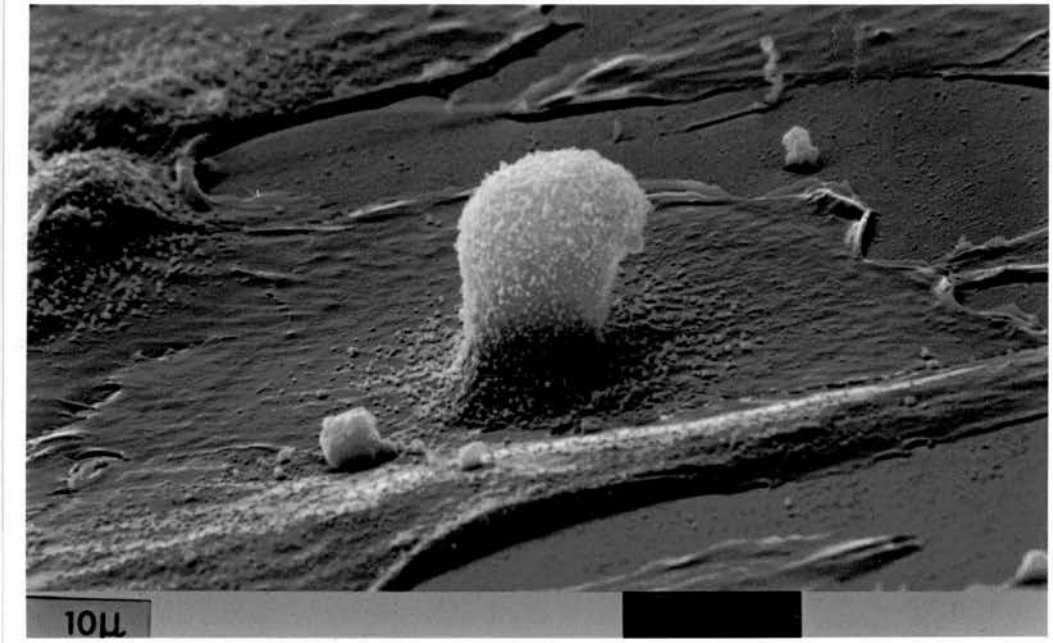
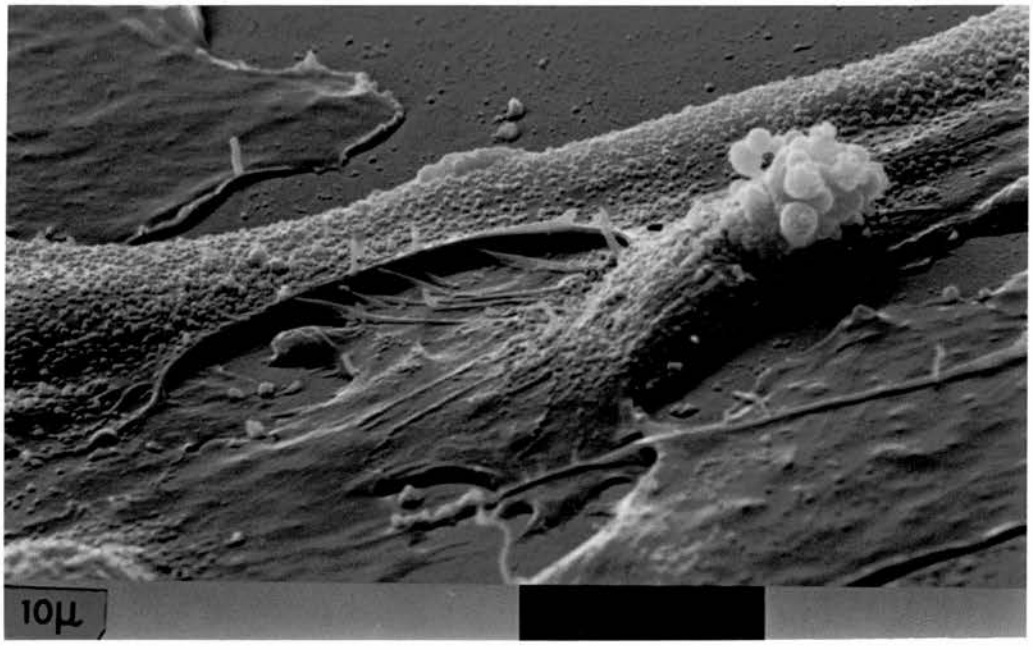


Fig. 36.

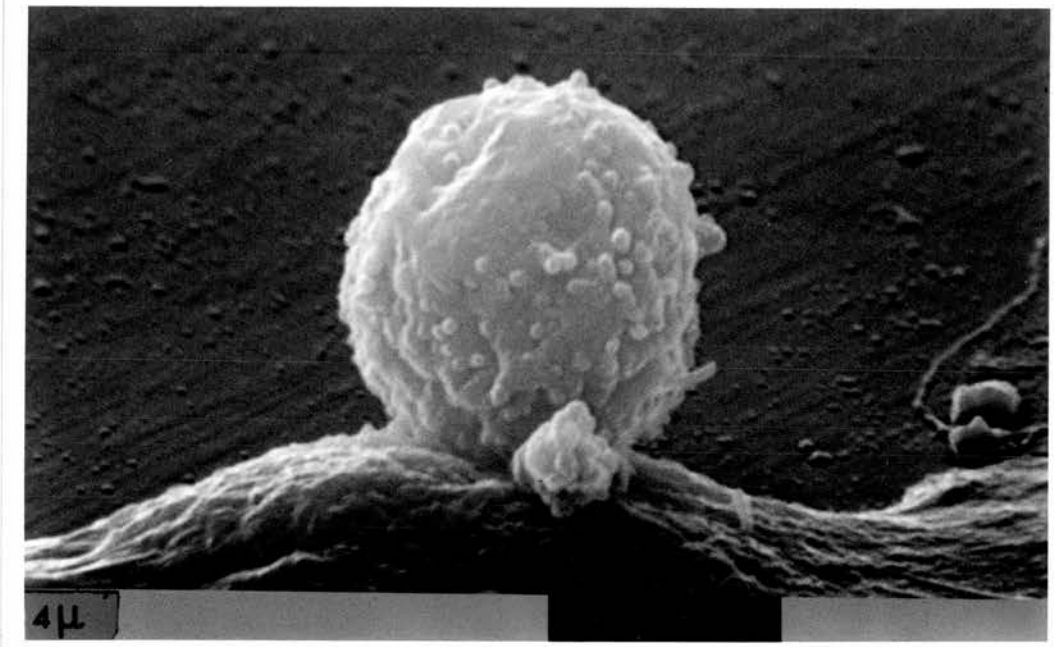
TC 67. Detail of a group of cells showing a group of smaller blebs, long filopodia, short microvilli and smooth cell surfaces.



Figs. 37 and 38.

TC 67. 7 days culture. Detailed views of cytoplasmic excrescences showing smooth areas and areas with short, stubby microvilli.

TC 67. 7 days culture. High power views of typical excrescences.



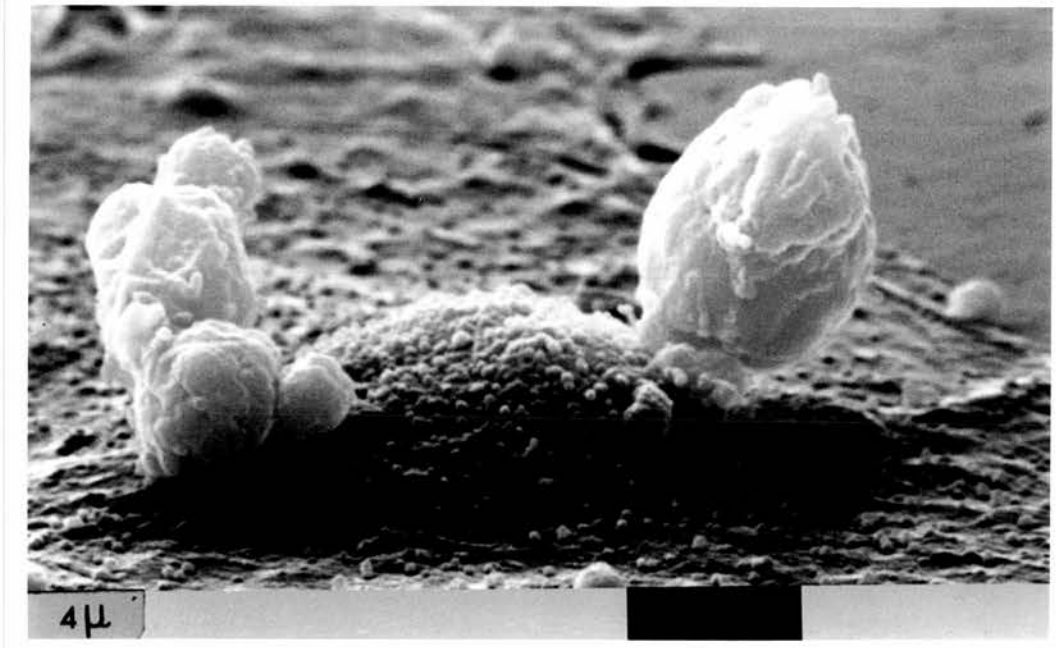


Fig. 39.

TC 76. Highly elastotic anaplastic carcinoma later shown to have an apocrine component (See Section III p.143).

7 days culture. Cells from this tumour formed closely knit epithelial colonies demonstrating a wide variety of surface features.

General view of cell colony.

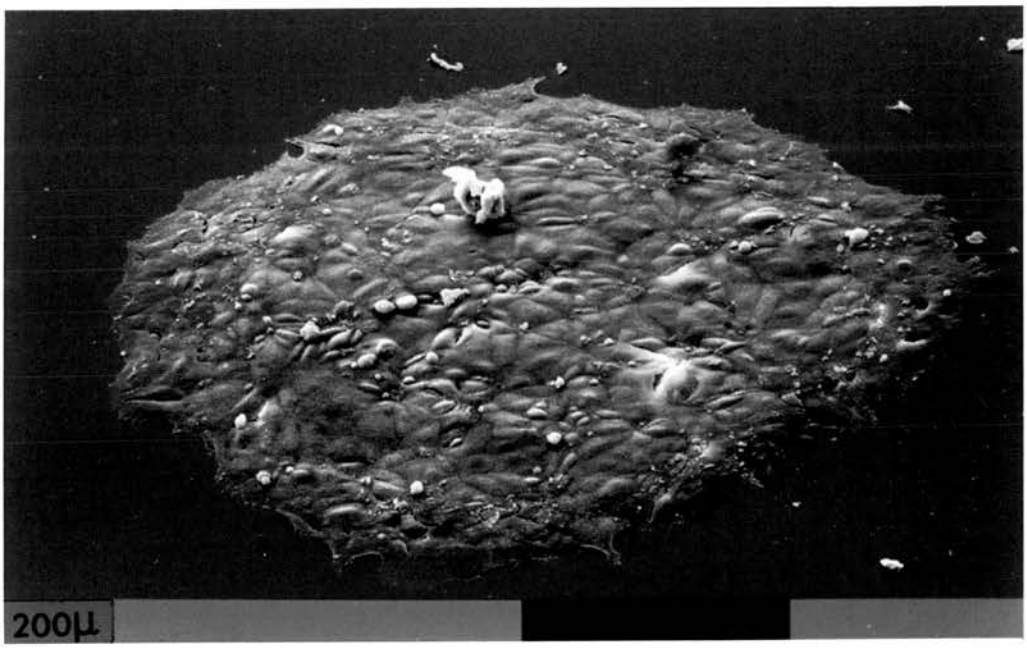


Fig. 40.

A cell showing a variety of excrescences on its surface.

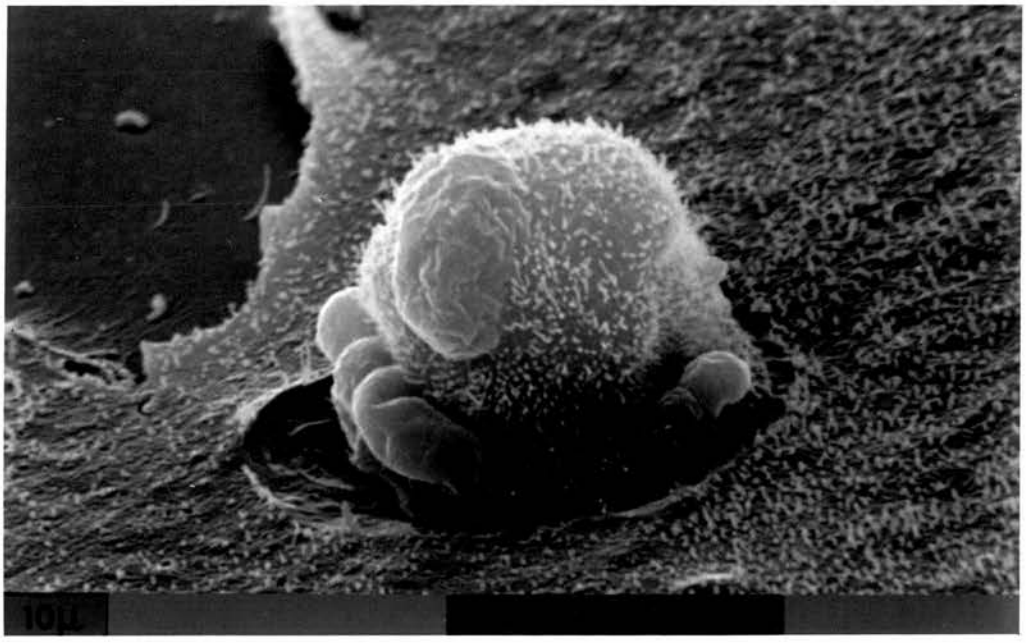


Fig. 41.
TC 76. 7 days culture. A small group of cells showing a variety of surface features.

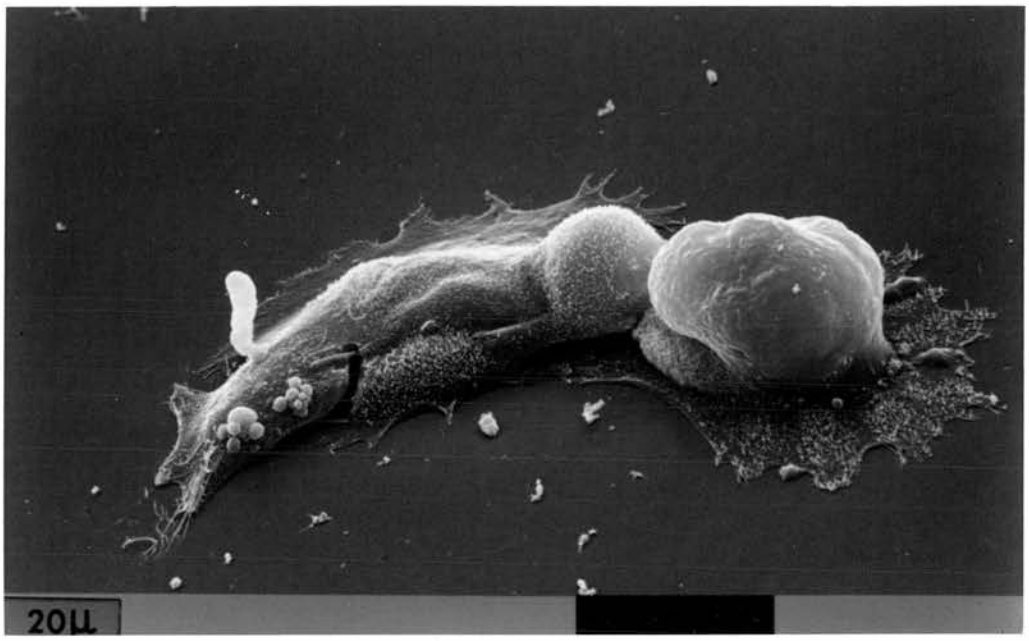


Fig. 42.
TC 76. An isolated cell with long filopodia and well developed lamellipodia (membranes).

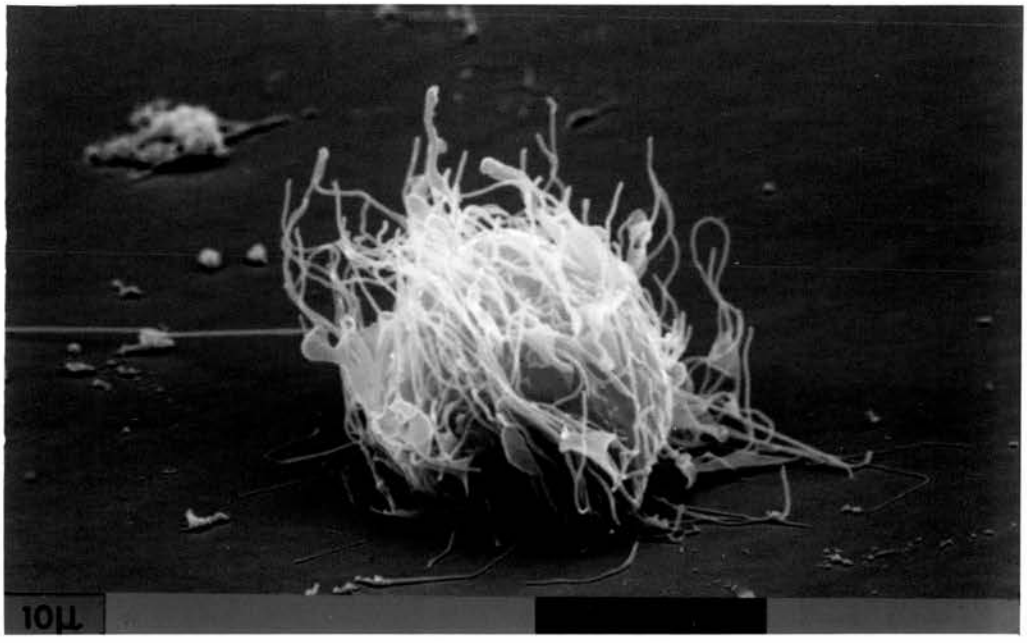


Fig. 43.

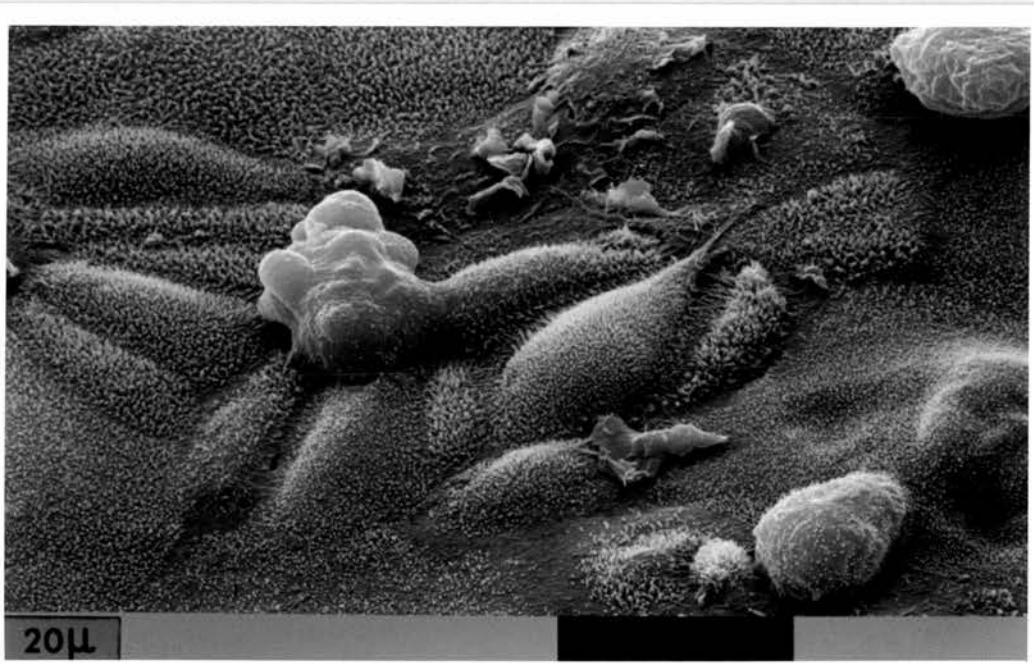
TC 76. 7 days culture. Two cytoplasmic excrescences, one relatively smooth and one covered in short microvilli, emerging from the surface of the cell sheet.

Fig. 44.

TC 76. 7 days culture. General view of an area showing cells covered with short microvilli, smooth excrescences, ruffled membranes and interdigitating filopodia.

TC 76. 7 days culture.

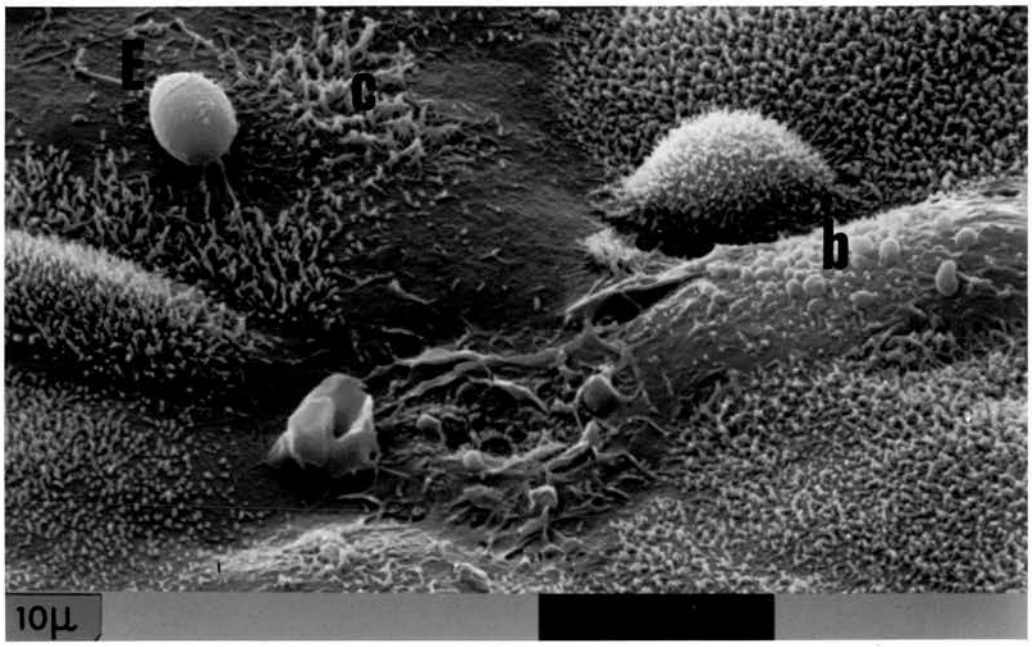
TC 76. 7 days culture.



Figs. 45 and 46.

TC 76. Detailed views of cell surface features.

TC 76. 7 days



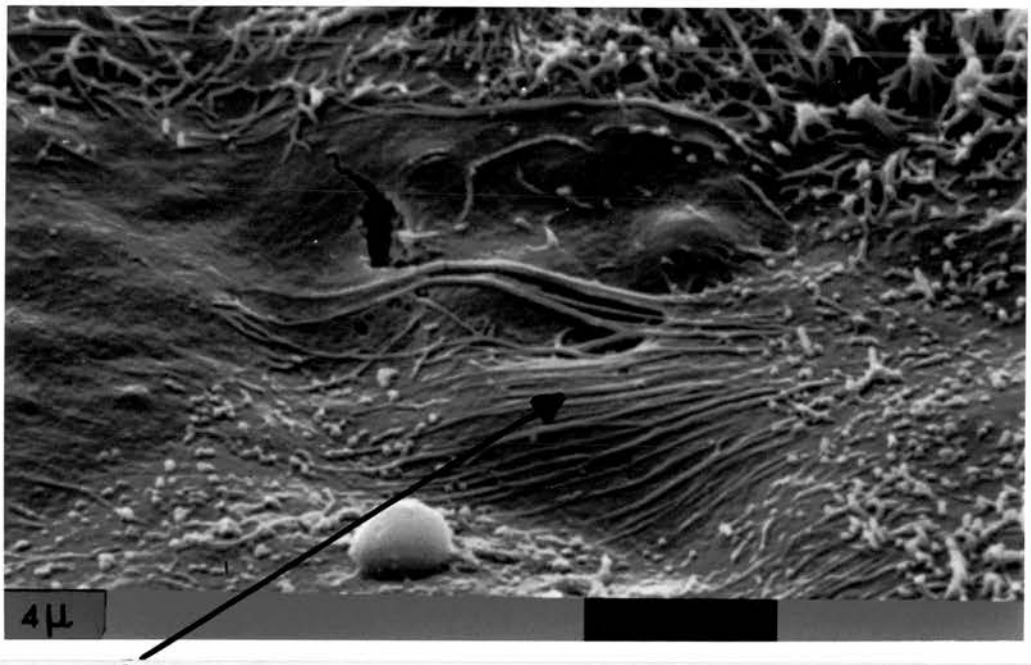
c Clumped microvilli

E Excrescence

m Circular membrane

b Blebs

TC 76. 7 days



Long, interdigitating filopodia on cell surfaces

c Clumped microvilli

Fig. 47.

TC 76. Detailed view of cytoplasmic 'crater' approximately 5 μm in diameter which had possibly been left after a 'bleb' had been extruded from the surface of the cell (pp 66).

TC 76. Cytoplasmic "crater", approximately 5 μm in diameter.

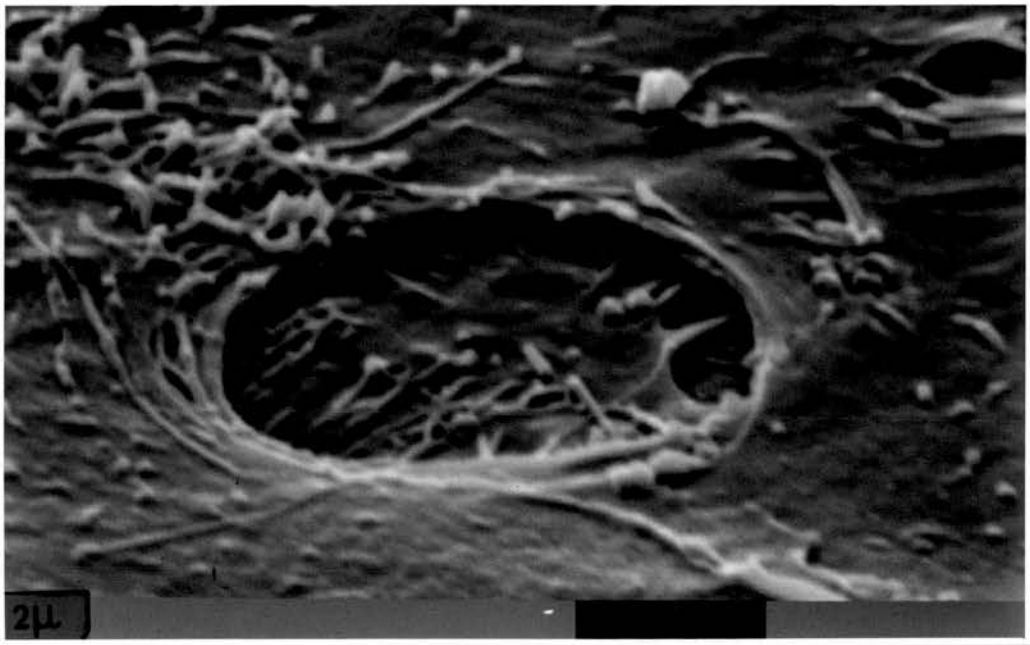
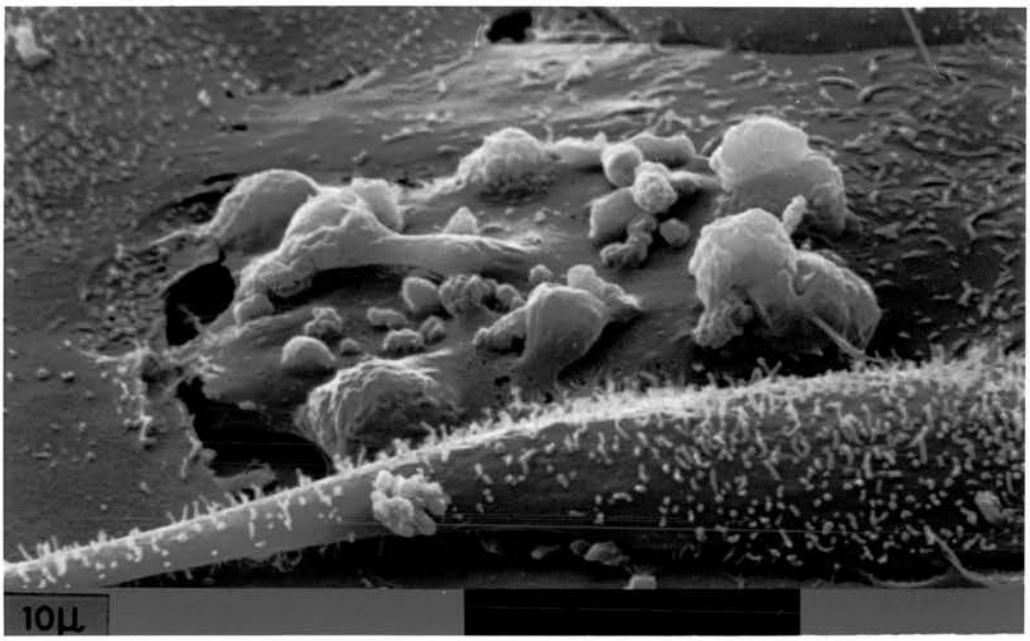


Fig. 48.
TC 77. Anaplastic carcinoma. Grade I elastosis. 7 days culture. General view of a 'blebbing' elastotic cell. The blebs vary in size between 1 µm and 5 µm.

Fig. 49.
TC 77. 7 days culture. Cells with many smaller blebs and also some very elongated excrescences, one approximately 10 µm long.

TC 77. 7 days culture. A variety of surface features.



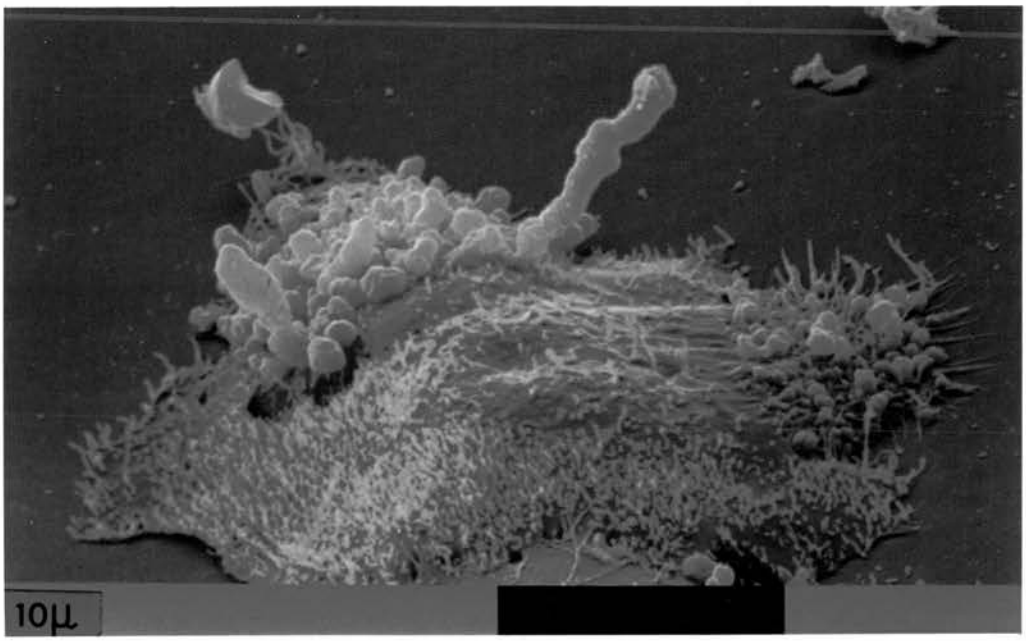
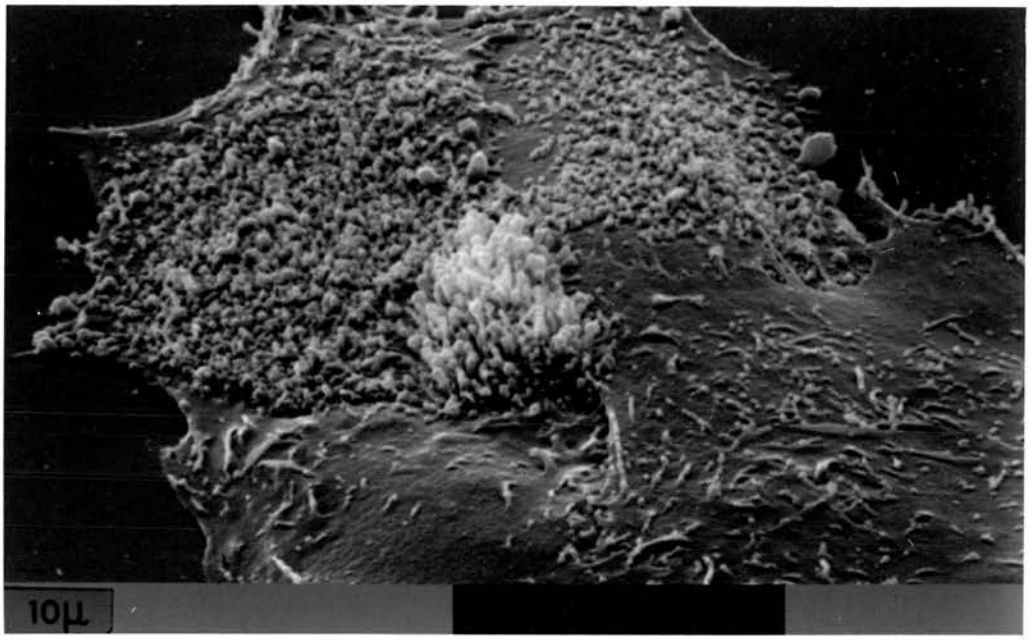


Fig. 50.
TC 77. 7 days culture. Cells with microvilli, blebs and ruffled membranes on their surfaces.

Fig. 51.
TC 77. 7 days culture. Detailed view of a cell covered with microvilli and blebs 1µm → 4 µm in diameter.

TC 77. 7 days culture. A variety of surface features.



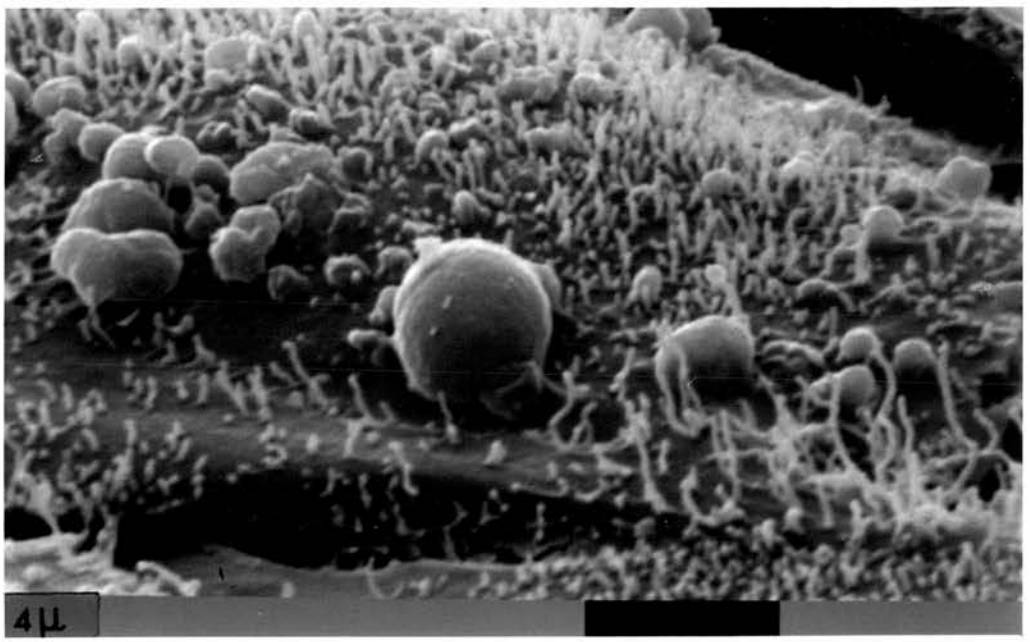


Fig 52.

TC 77. 7 days culture. General view of a large (≃ 12 µm diameter) smooth cytoplasmic excrescence.

Fig. 53.

TC 77. 7 days culture. Detailed view of  indicating continuity between the excrescence and the immediate surroundings.

TC 77. 7 days culture. A variety of surface features.

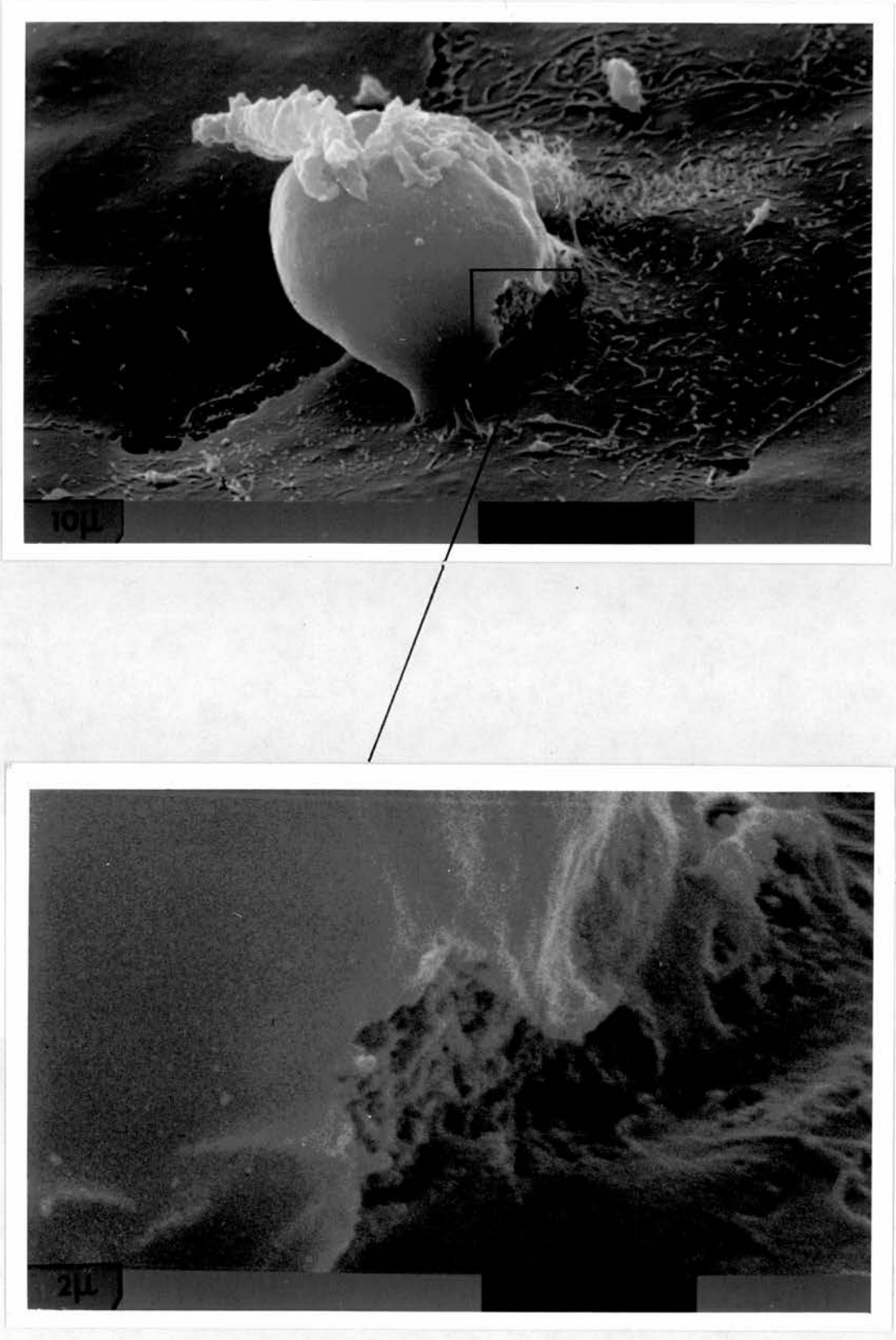


Fig. 54.

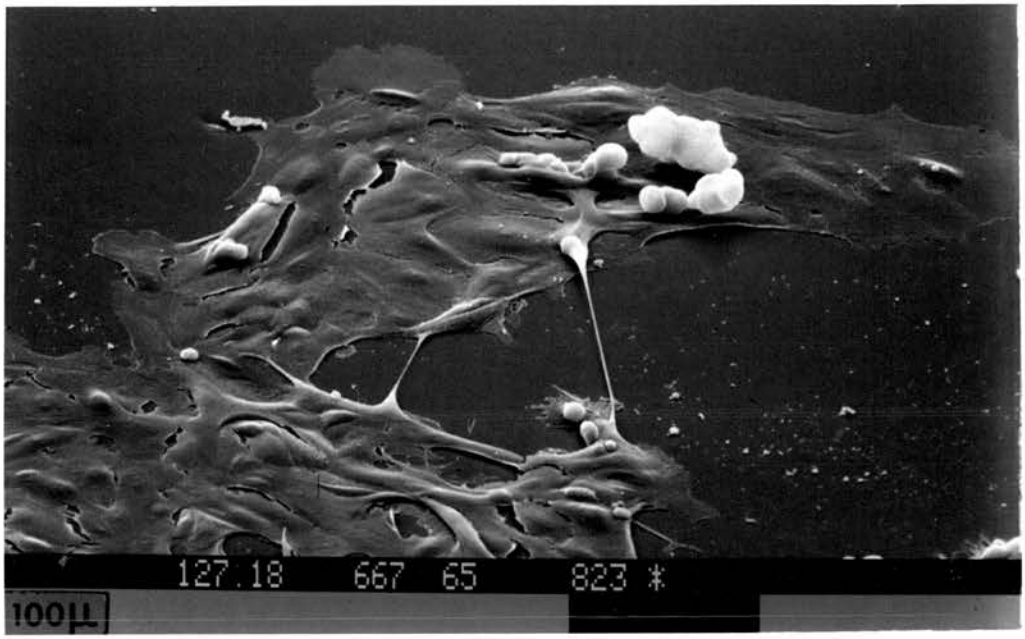
TC 82. Highly elastotic anaplastic carcinoma. 20 days culture.

General view of epithelial cell pavements with intercellular 'bridges'.

Fig. 55.

TC 82. Detailed view of an intercellular bridge.

TC 82. Highly elastotic anaplastic carcinoma later shown to have an apocrine component. (See Section III p. 143).
Cells from TC 82 grew in flat sheets, often connected by intercellular bridges. 20 days culture.



TC 82. The intercellular bridges were not smooth but were covered with minute nodules, approximately 0.01 µm in diameter.

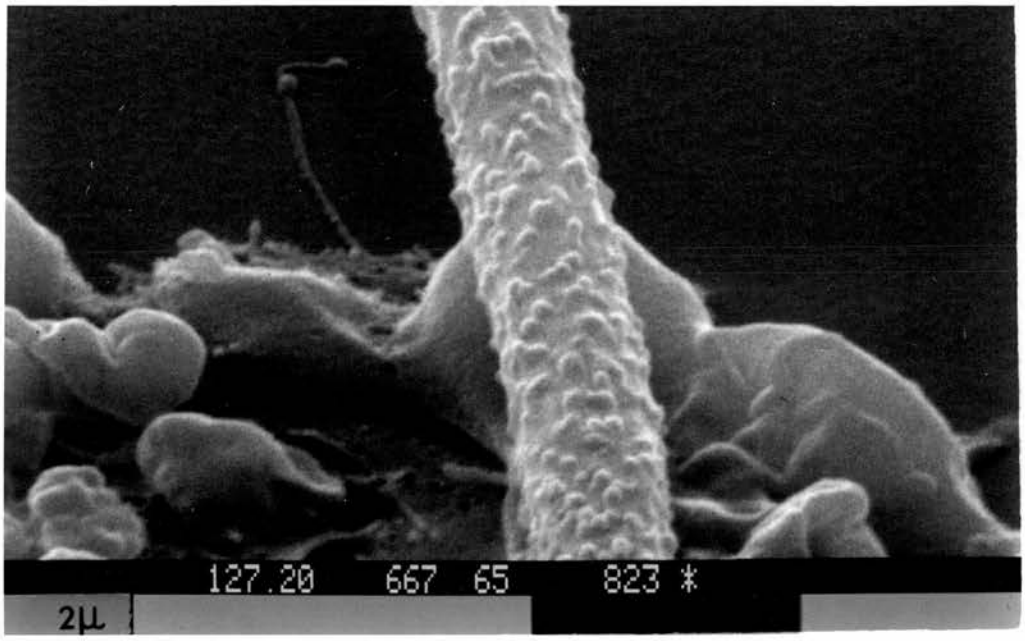


Fig. 56.

TC 82. 20 days culture.

At the edges of the cell groups the cells were often piled up on one another forming cords of cells several layers thick. Cytoplasmic excrescences were a common feature in these areas.

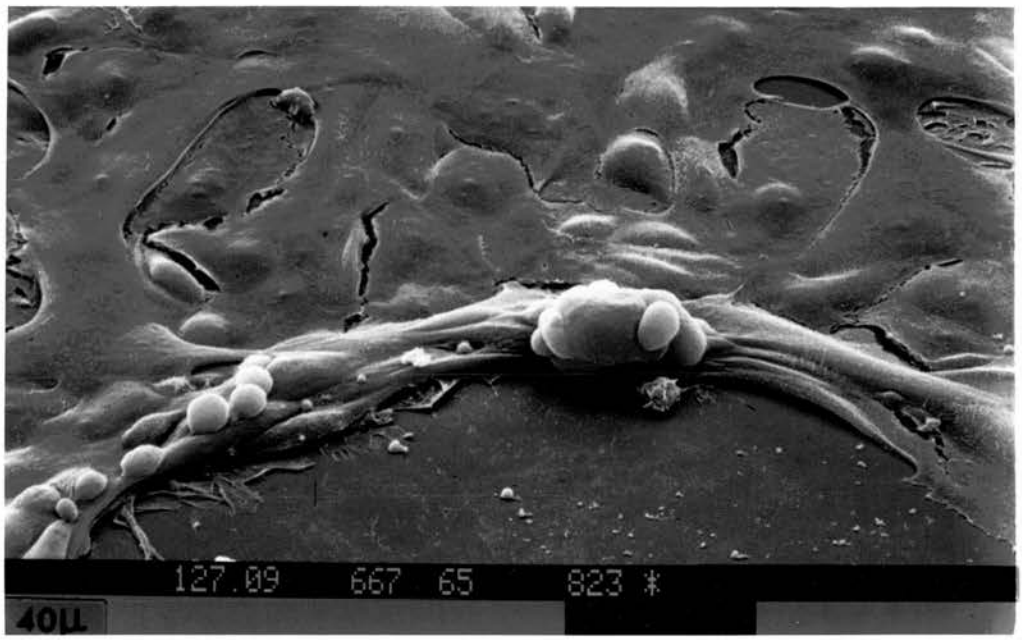


Fig. 57.

TC 82. The surface morphology of the excrescences varied considerably. Some were smooth, others had microvilli or longer filopodia, others appeared to have cytoplasmic ridges.

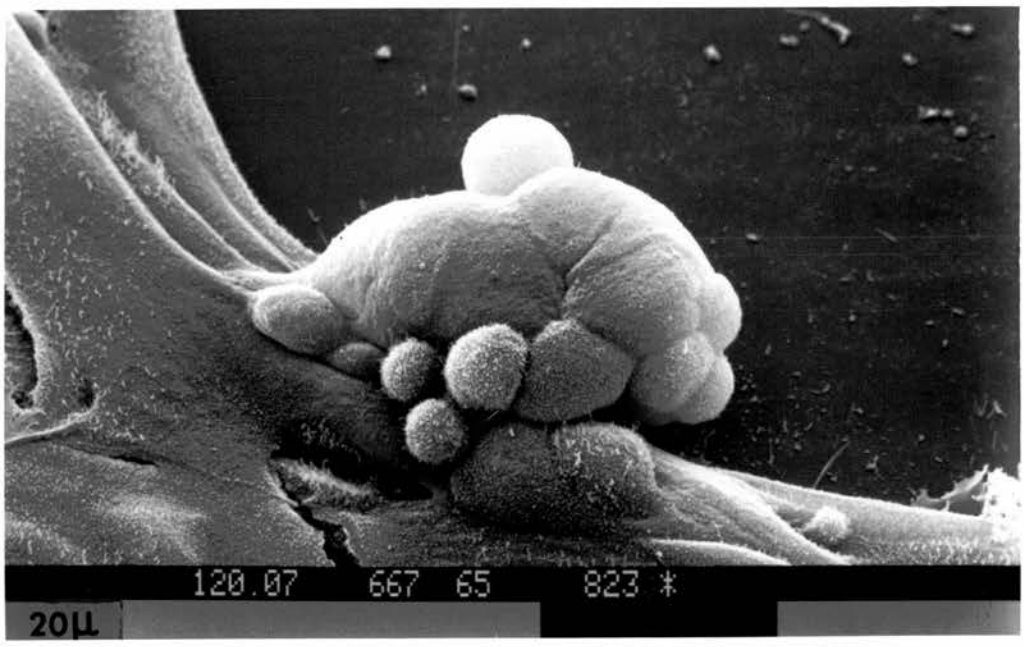


Fig. 58.

TC 82. 20 days culture. Detailed view of cytoplasmic excrescences found at the edge of the cell pavement (see Figs. 56 and 57). Some blebs were smooth, others were covered with microvilli.

TC 82. 20 days culture. Note variation in surface features.

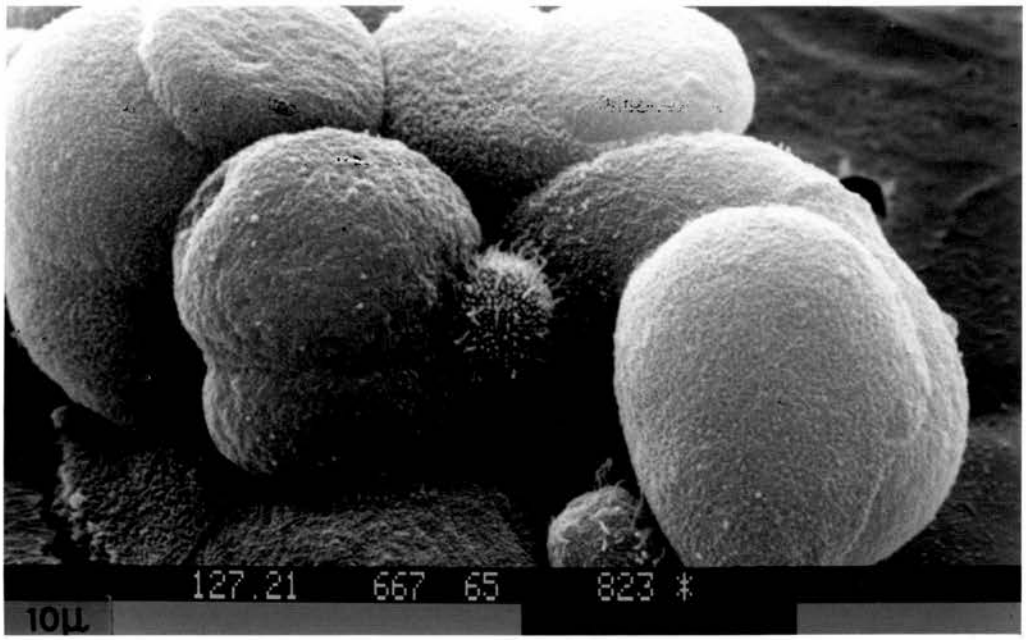


Fig. 59.

TC 82. Excrescence with stubby microvilli. 20 days culture.

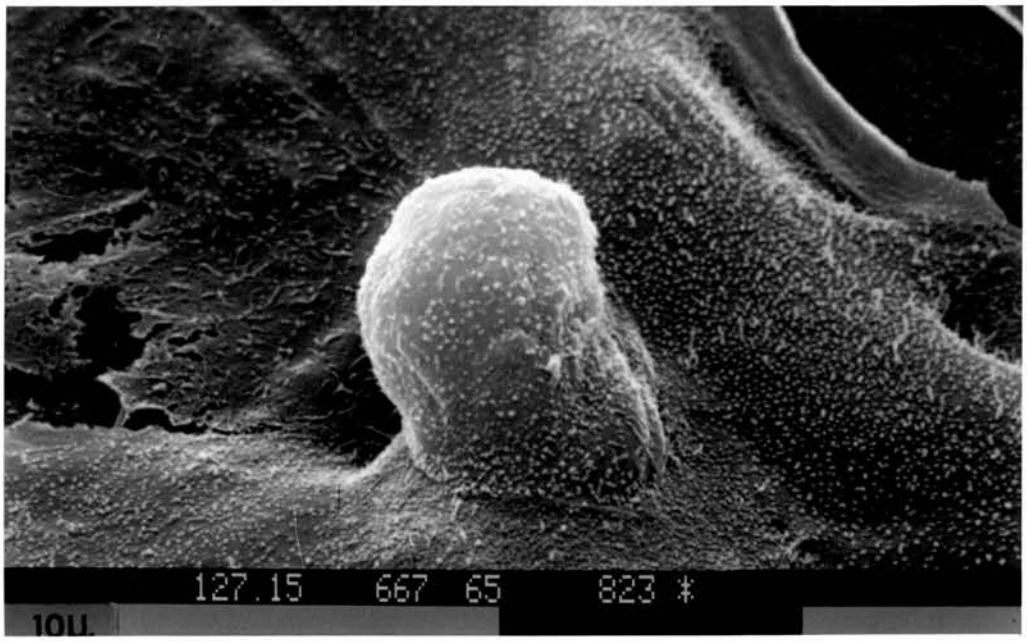


Fig. 60.

TC 82. 20 days culture. Excrescence or cell with ridges.

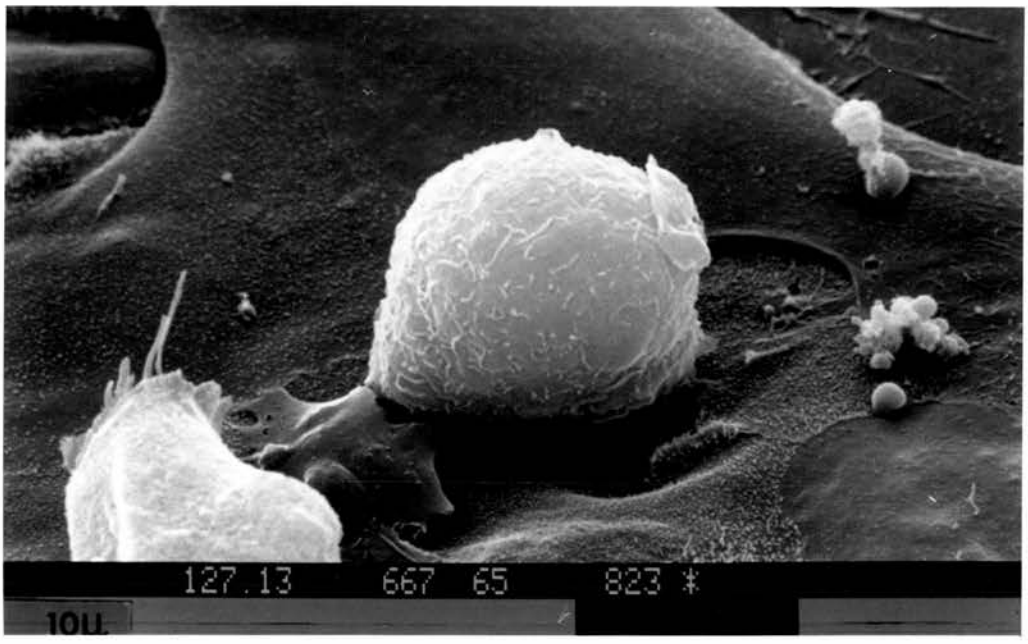


Fig. 61.

TC 82. 20 days culture. The variety of cell surface structures was remarkable. Some filopodia were 4 - 5 µm in length.

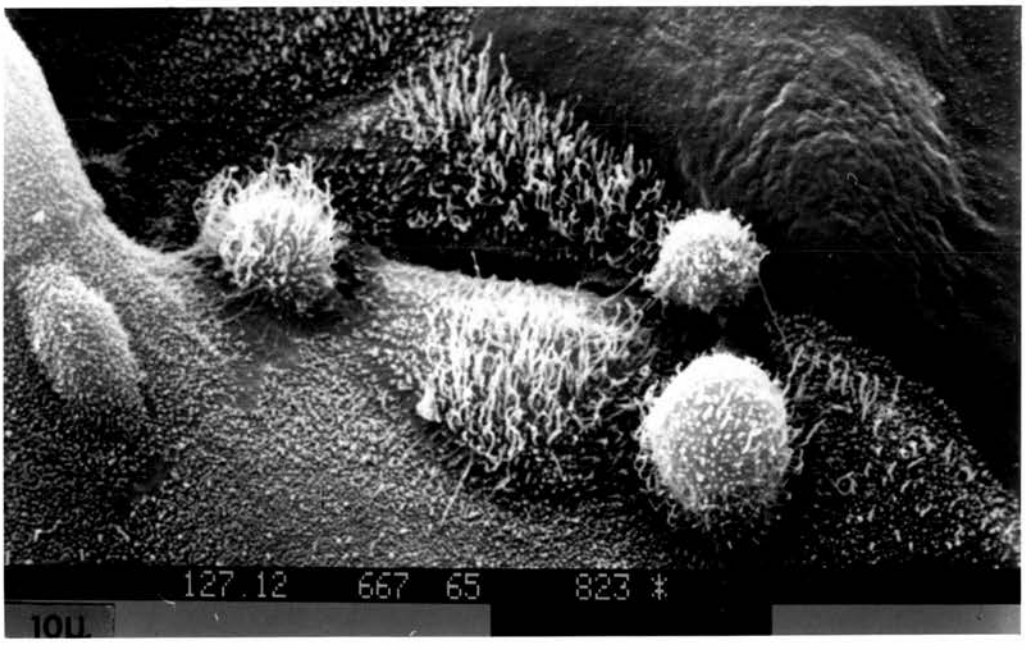


Fig. 62.

TC 82. Detailed view of a tuft of microvilli.

TC 82. 20 days.

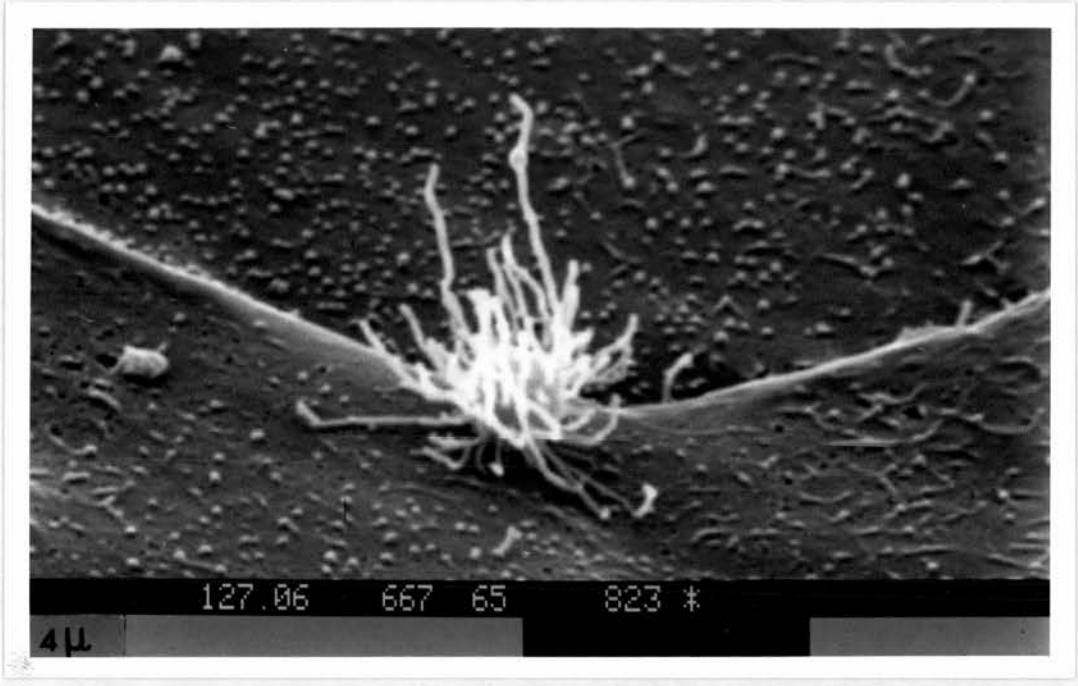


Fig. 63.
TC 82. Lamellipodia were also a feature. 20 days.

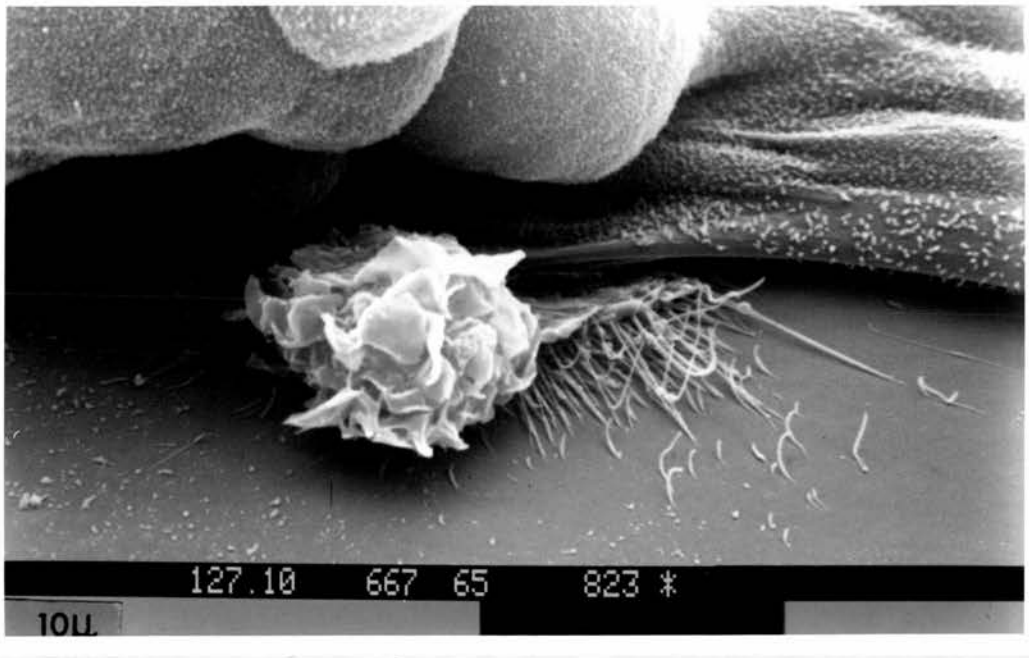


Fig. 65.

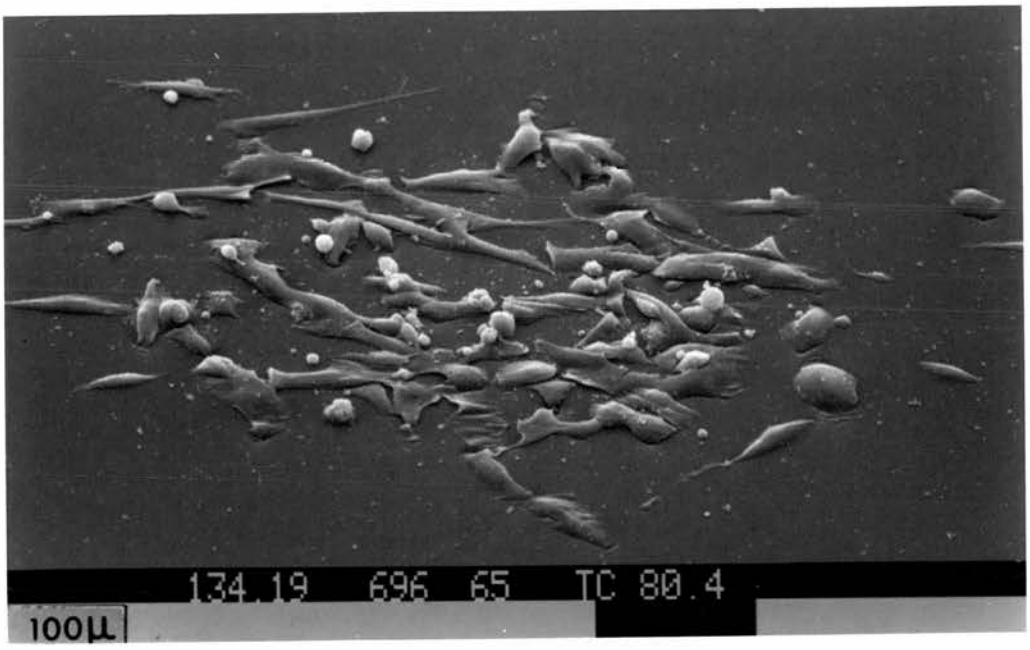
TC 80. 6 days culture. Detailed view of several large cytoplasmic excrescences emanating from one cell.

Fig. 64.

TC 80.

Highly elastotic anaplastic carcinoma. 6 days culture.

The overall appearance of the cells which grew from TC 80 was similar to those of TC 67 (pp 68). Once again cytoplasmic extrusions were a conspicuous feature. The surface characteristics of the extrusions were very varied.



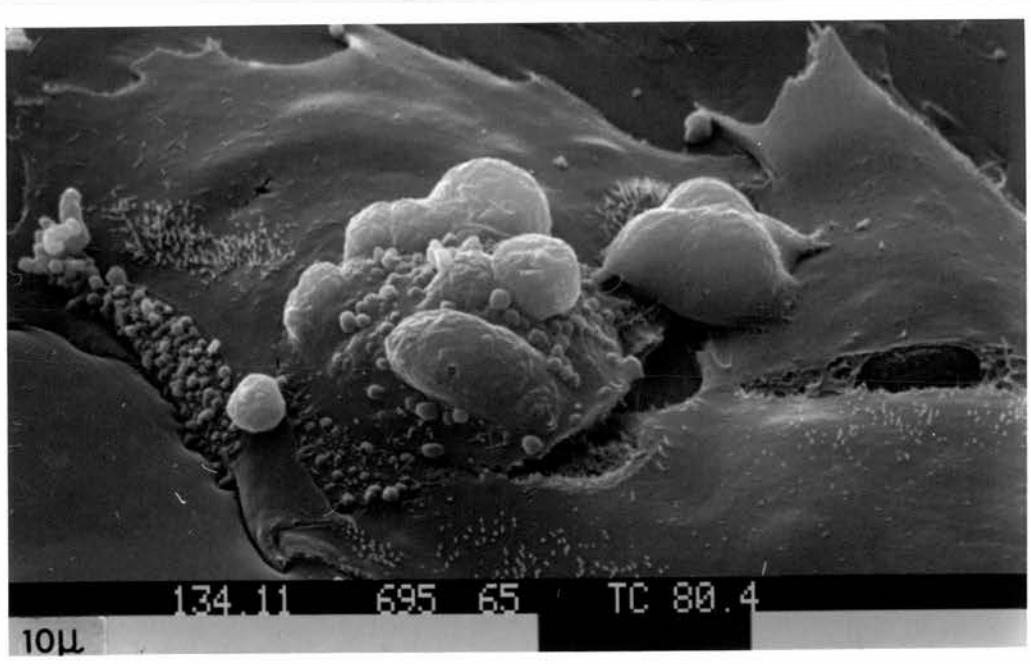


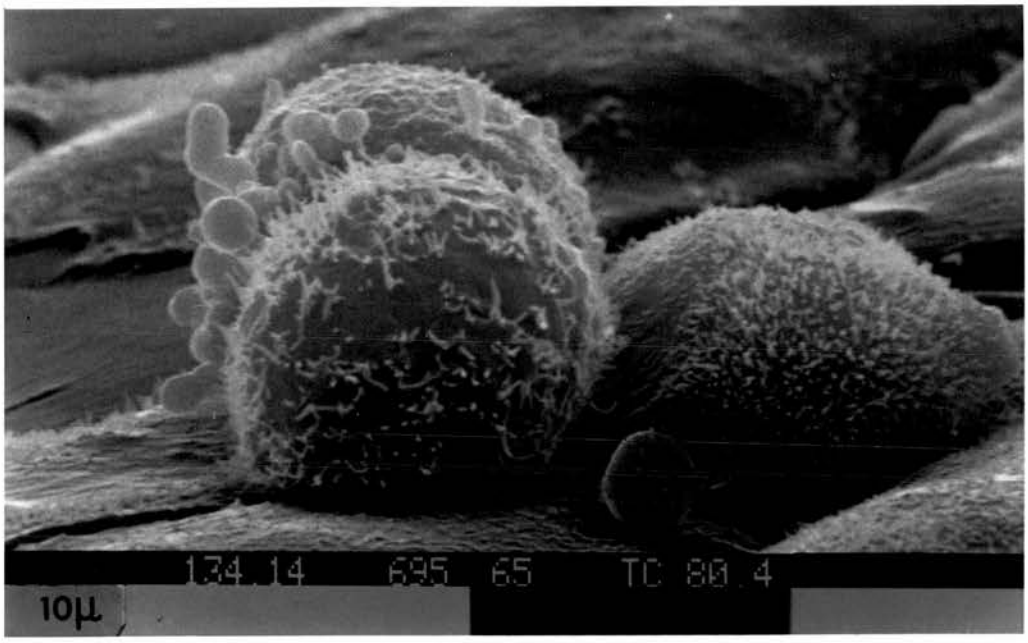
Fig. 66.

TC 80. 6 days culture. Blebs on microvilli covered cells.

Fig. 67.

TC 80. 6 days culture. Large, smooth excrescence or complete cell.

TC 80.



TC 80.

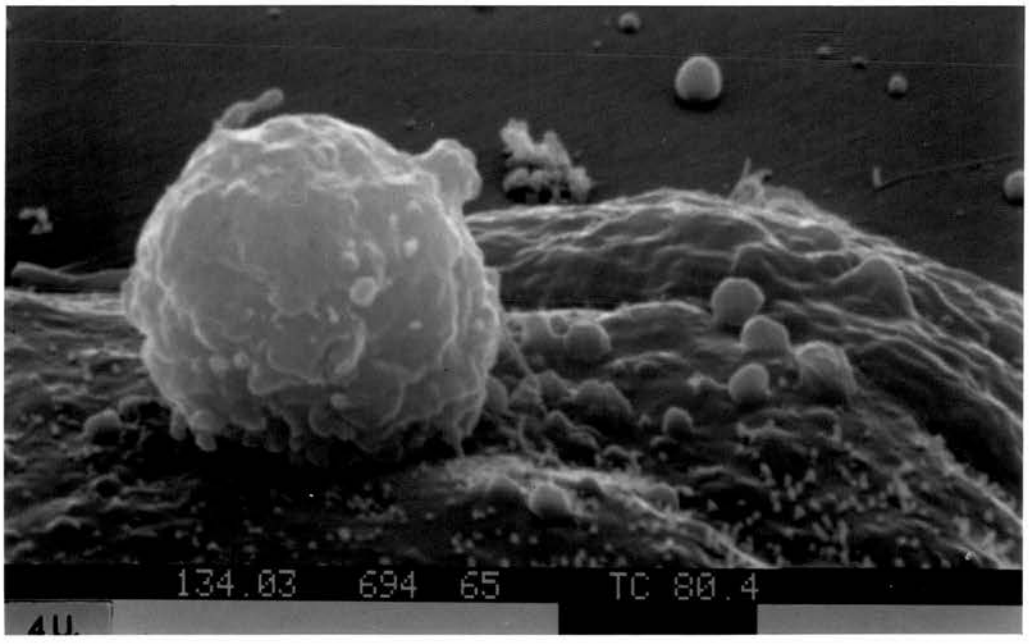


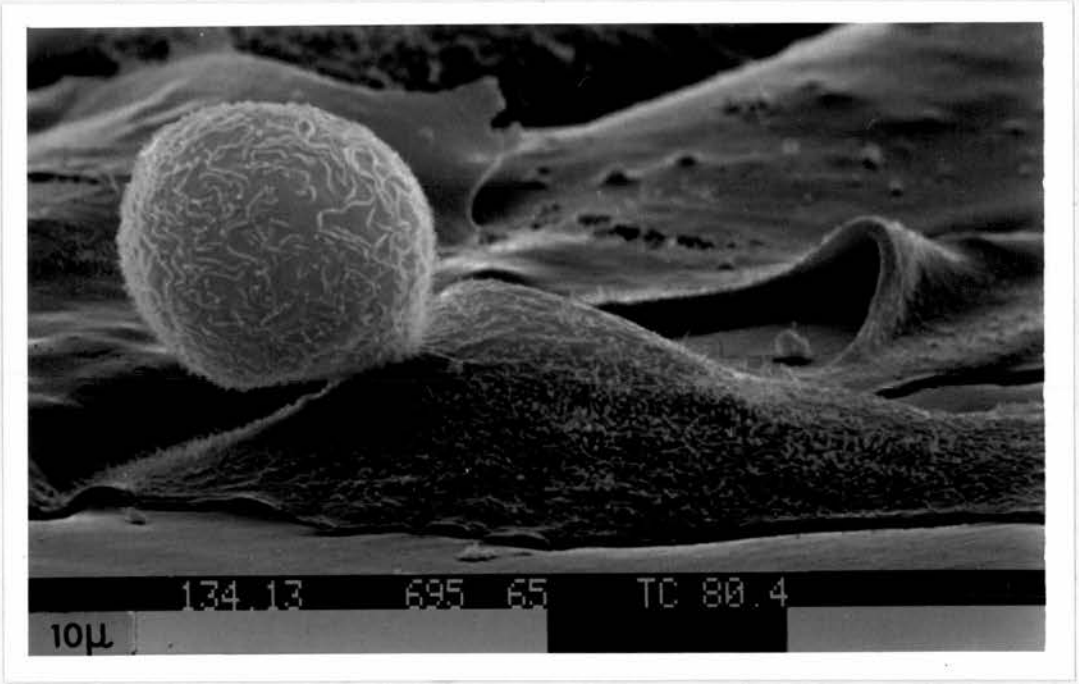
Fig. 68.

TC 80. 6 days culture. Cytoplasmic excrescence or whole cell covered in Lamellipodia.

Fig. 69.

TC 80. 6 days culture. A smooth cytoplasmic bleb, approximately 5 µm in diameter, emerging from a cell covered with short microvilli.

TC 80.



TC 80.

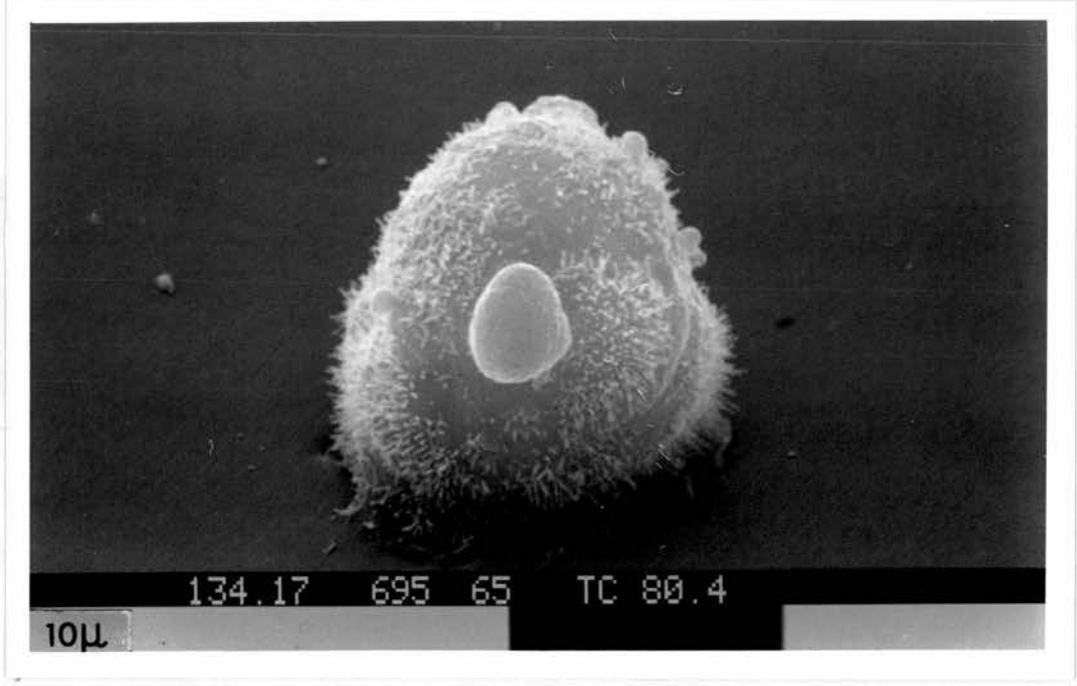


Fig. 71.

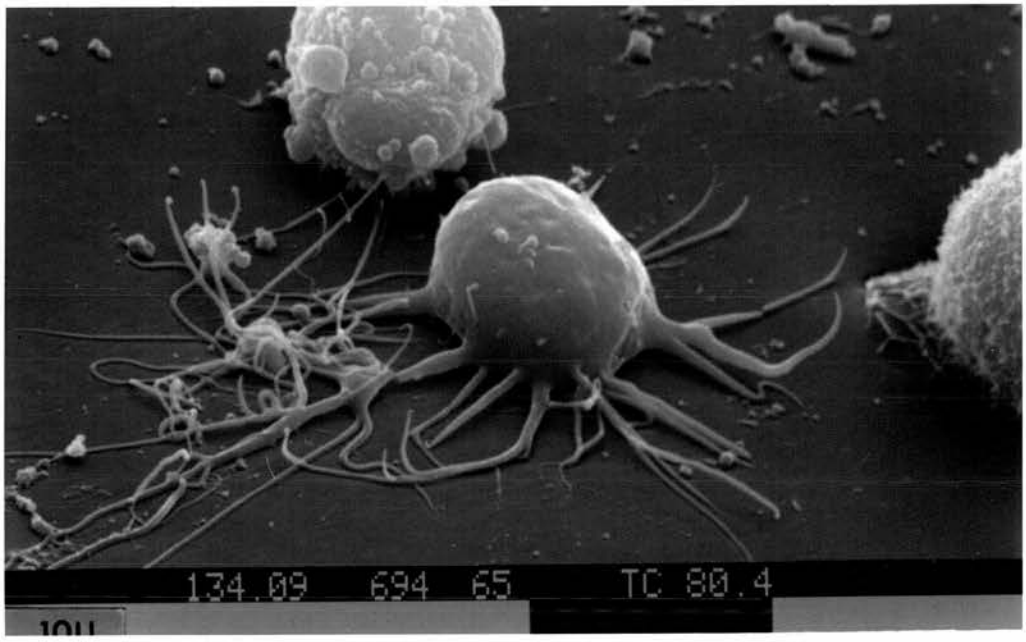
TC 80. 6 days culture. Detailed view of filopodia.

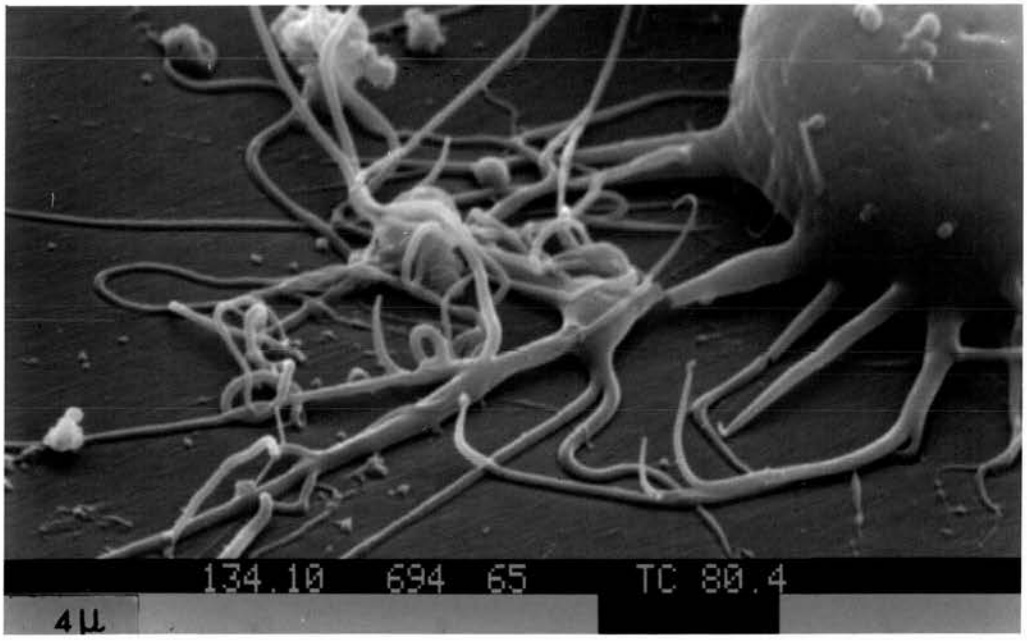
Fig. 70.

TC 80.

6 days culture.

Occasionally cells with long, thick, sometimes branched cytoplasmic extensions were seen. These often tangled into networks.





The significance of the many and varied surface structures seen in cultures of highly elastotic breast tumours, using the scanning electron microscope, is not known. The use of techniques allowing the consecutive examination of the same piece of tissue by scanning electron microscopy, light microscopy and transmission electron microscopy [28] would contribute a great deal to the understanding of the phenomena observed.

Fig. 72.

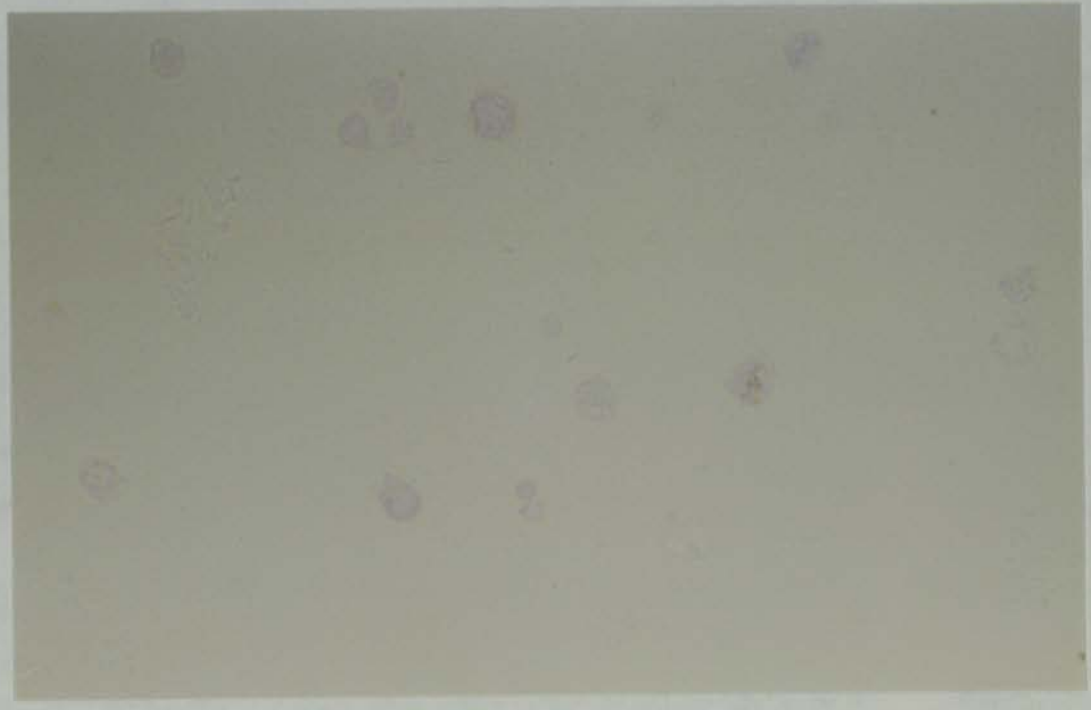
TC 67. Lobular carcinoma with a high degree of elastosis. Cytoplasmic 'blebs' stained positively (purple) for the presence of elastin.

iii) Identification of Extruded Material.

In order to determine what the extruded material comprised 'droplet' preparations (pp 34) were made, stained for the presence of elastin with the Gomori aldehyde fuchsin technique, and examined. A regular finding was the presence of a layer or network of purple, elastin positive material around each cytoplasmic 'bleb'.

The Gomori aldehyde fuchsin technique was originally chosen as, unlike many of the other elastin specific stains which render the elastin black, e.g. Verhoff and Weigert, Gomori's aldehyde fuchsin technique stains the elastin purple. Elastin may therefore be easily distinguished from cell boundaries on the basis of colour when viewed with a phase contrast optical system.

TC 67. Droplet preparation. Gomori aldehyde fuchsin, no counterstain. Original magnification X 144



Figs. 73 and 74.

TC 67. Droplet preparation. Gomori aldehyde fuchsin/tartrazine.

Original magnification X 360

Note: The addition of the counterstain renders the purple dark brown to black.

Figs. 75 and 76.

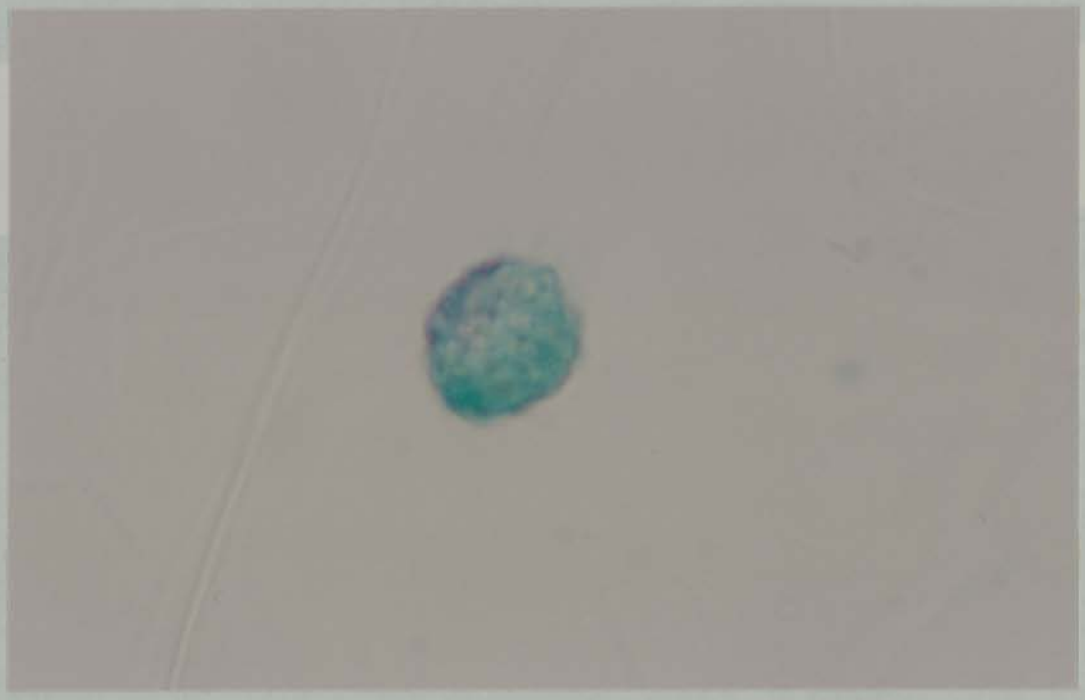
TC 67. Gomori aldehyde fuchsin/tartrazine. Note the absence of elastin positive material around some of the cytoplasmic 'blebs'. Original magnification X 360

Figs. 77 and 78.

TC 77. Anaplastic carcinoma. Grade I elastosis. Droplet preparations.

TC 77. Gomori aldehyde fuchsin/light green. Gomori positive material forms a network around the 'bleb'.

Original magnification X 360



Original magnification X 360

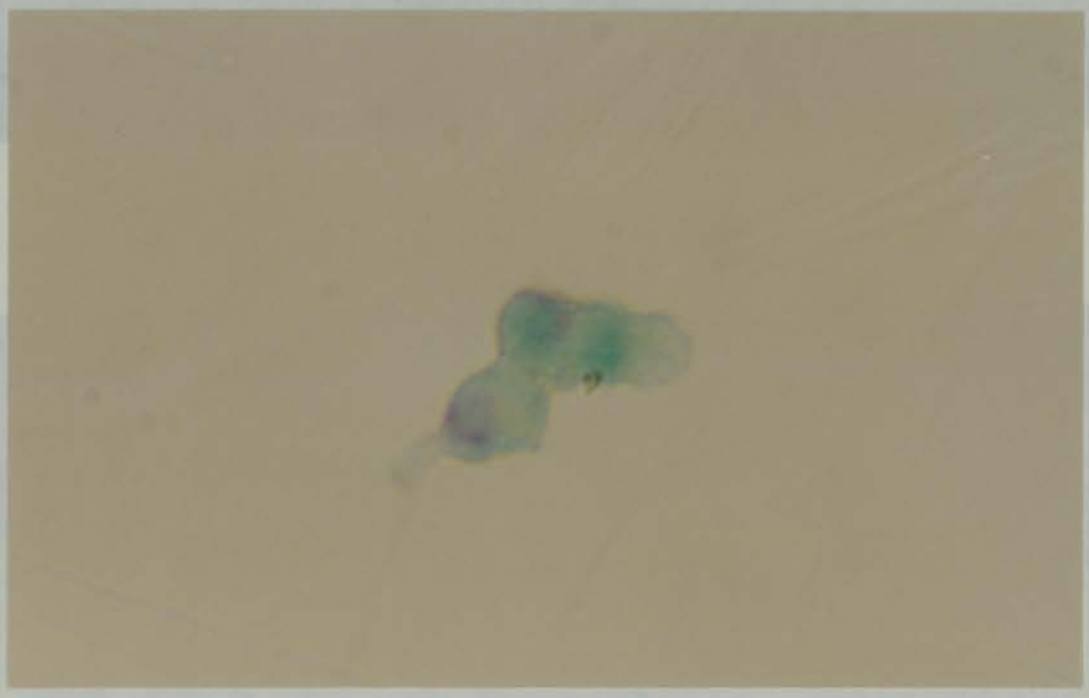


Fig. 79.

TC 76. Anaplastic carcinoma. Grade II elastosis. Droplet preparation.

Fig. 80.

TC 86. Anaplastic carcinoma. Grade II elastosis. Droplet preparation.

The addition of a nuclear stain sometimes revealed the presence of whole cells or small groups of cells surrounded by elastin.

TC 76. Gomori aldehyde fuchsin/haematoxylin/tartrazine.

Original magnification X 360

There were also collections of fibrillar, Gomori positive material which were not associated with any cytoplasm.

TC 86. Original magnification X 144

It/

Fig. 81.

TC 82. Anaplastic carcinoma. Grade II elastosis. Droplet preparation.

It is possible that material of this type was 'carried over' from the original biopsy as, unlike the extrusion of the cytoplasmic blebs, there was no evidence on the basis of light or scanning electron microscopy to support the formation of fibres of elastin 'in vitro'.

A further structure in many of the preparations which apparently stained positively for the presence of elastin was a membranous "bag" containing purple granules and often a small quantity of cytoplasm.

TC 82. Gomori aldehyde fuchsin/light green.
Original magnification X 144

The significance of these structures is not known although, as Mast cell granules also stain purple with the Gomori aldehyde fuchsin technique, the possibility that these structures represent such granules cannot be ignored. Examination of sections of one of the highly elastotic tumours from which this type of structure arose (TC 67) revealed a significant number of mast cells within the carcinomatous area.

Confirmation/

Confirmation of the origin of the granules within mast cells might come with positive staining using Csaba's Alcian blue - safranin method.[34]

Droplet preparations made from 6 highly elastotic tumour cultures stained positively for the presence of elastin. TC67, 68, 76, 77, 82 and 86. No attempt was made to make droplet preparations from 8 cultures (6 prior to biopsy number TC 67 and also TC 69 and TC 80) and those slides prepared from TC 83 proved to be inadequately dried and the cellular material was lost from the slides during histological processing.

Fig. 82.

TC 76. Anaplastic carcinoma. Grade II elastosis.

The blebs seen here, stained strongly purple with Gomori's aldehyde fuchsin, are the equivalent of the refringent and greyish excrescences depicted in Figs. 8, 9 and 10 (pp 52 and 53) also Figs. 26, 27 and 28 (pp 61 and 62).

iv) Identification of elastin "in the flask".

Early attempts to demonstrate elastin in cultures of breast cancer cells were unsuccessful. In each case cultures of at least four weeks duration were selected for fixation and staining as it was surmised that late cultures would be more likely to include newly synthesised elastica.

Following the demonstration of Gomori positive material which had been extruded from cells early in their culture history, cells from TC 76, a highly elastotic anaplastic carcinoma, were fixed with neutral buffered formalin at 12 days culture and stained "in the flask" with Gomori's aldehyde fuchsin. Positive staining for elastin was particularly noticeable in the cytoplasmic and cellular excrescences, thus confirming the evidence of the droplet preparations.

TC 76. 12 days culture. Gomori's aldehyde fuchsin.
No counterstain. Original magnification X 150.

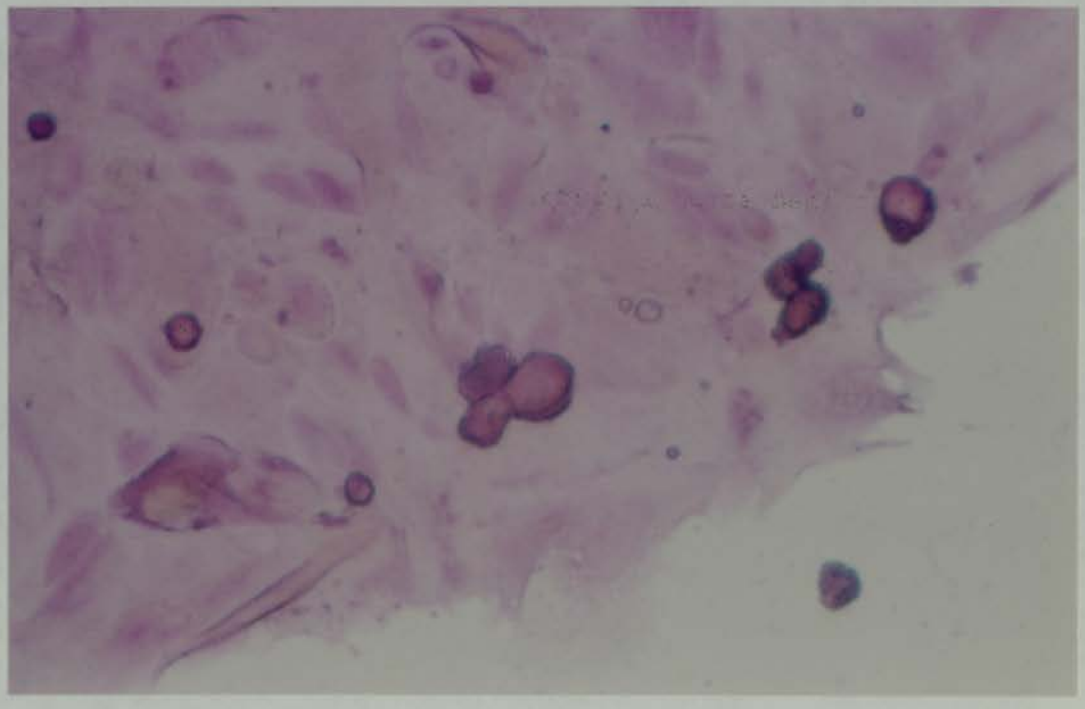


Fig. 83.

TC 76. Anaplastic carcinoma. Grade II elastosis.

The blebs seen here, stained strongly purple with Gomori's aldehyde fuchsin, are the equivalent of the refringent and greyish excrescences depicted in Figs. 8, 9 and 10 (pp 52 and 53) also Figs. 26, 27 and 28 (pp 61 and 62).

TC 76.

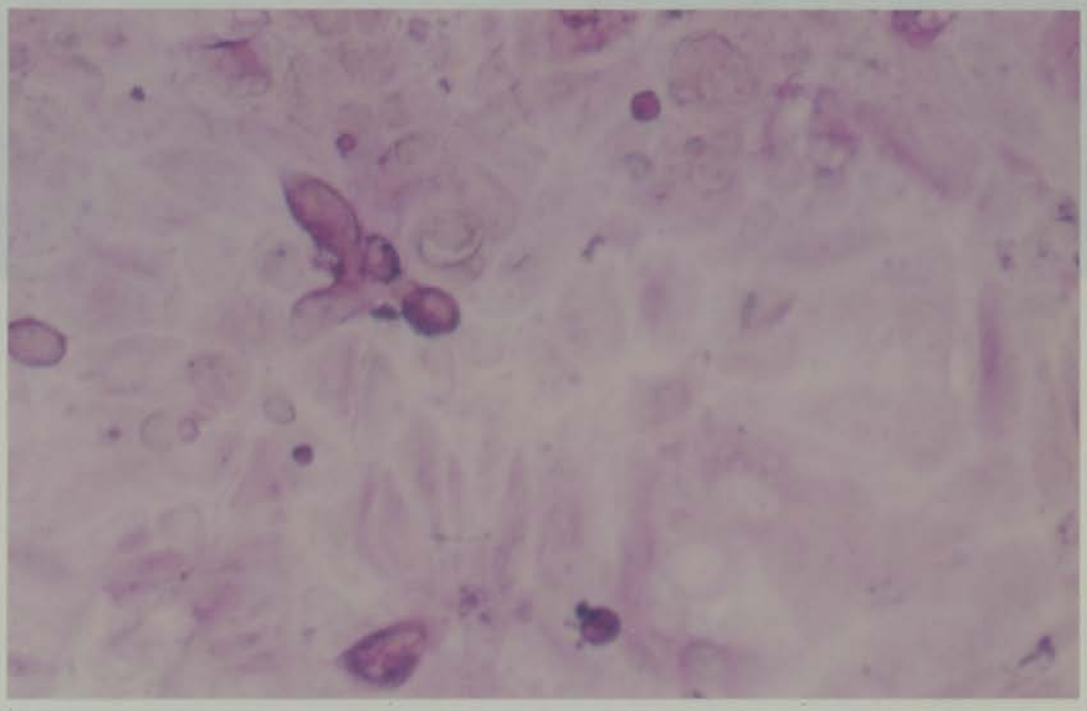


Fig. 84.

TC 83. Anaplastic carcinoma. Grade II elastosis. Myofibroblasts at relatively low concentration lining up in parallel rows.

Fig. 85.

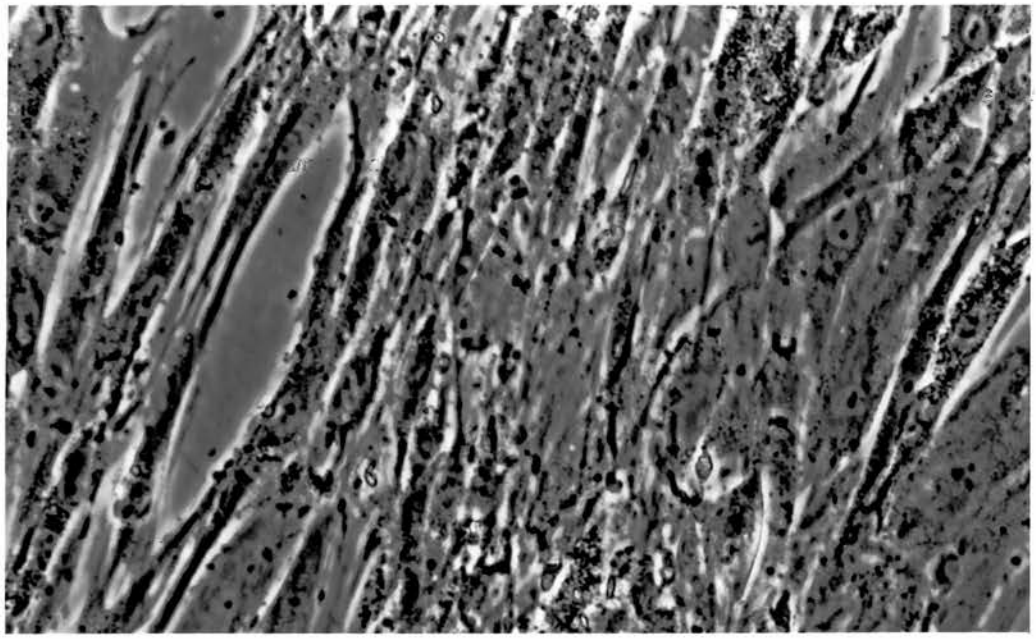
TC 77. Anaplastic carcinoma. Some elastosis. Myofibroblasts at high concentration piled up on one another.

v) The Growth of Myofibroblasts.

One further cell type which grew in cultures of both highly elastotic and non-elastotic tumours was a cell designated, on morphological, behavioural and ultrastructural grounds, a myofibroblast. The occurrence of this type of cell is given in Tables II, III and IV, and the cell is further alluded to in Sections II (p. 116) and V (p. 180).

In cultures of elastotic tumours myofibroblasts grew as elongated or stellate cells which, at low concentration, lined up parallel to one another, but at higher concentrations formed many-layered mats.

TC 83 21 days culture.



TC 77. 11 days culture.

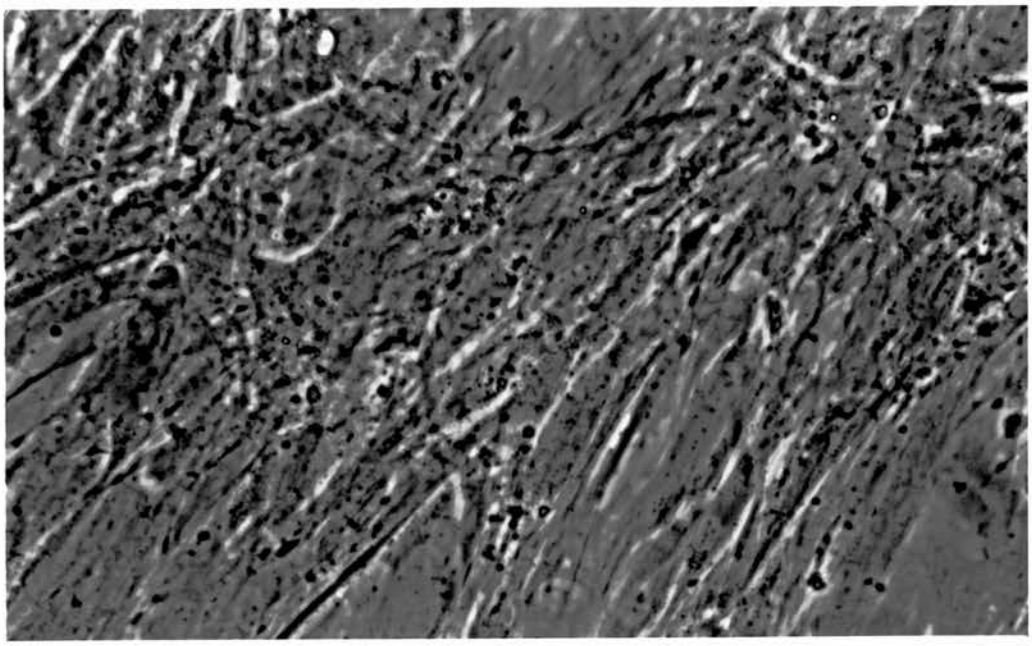


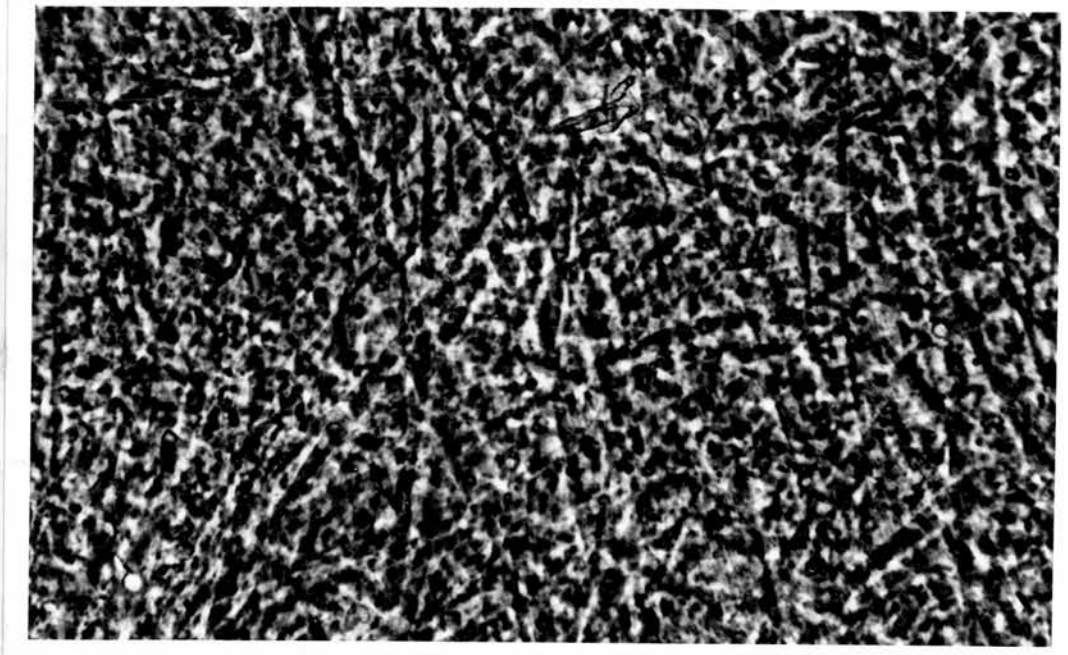
Fig. 86.

TC 76. Anaplastic carcinoma. Grade II elastosis. Myofibroblasts in a many layered mat.

Fig. 87.

TC 77. Anaplastic carcinoma. Some elastosis. Myofibroblasts contracted into a cellular ridge with associated fibrillar material.

TC 76 30 days culture.



Their most characteristic feature was an apparent contraction into ridges and lumps of cells, conspicuous even on macroscopic examination of the flasks. Associated with the ridges there was often fibrillar material.

TC 77. 56 days culture.

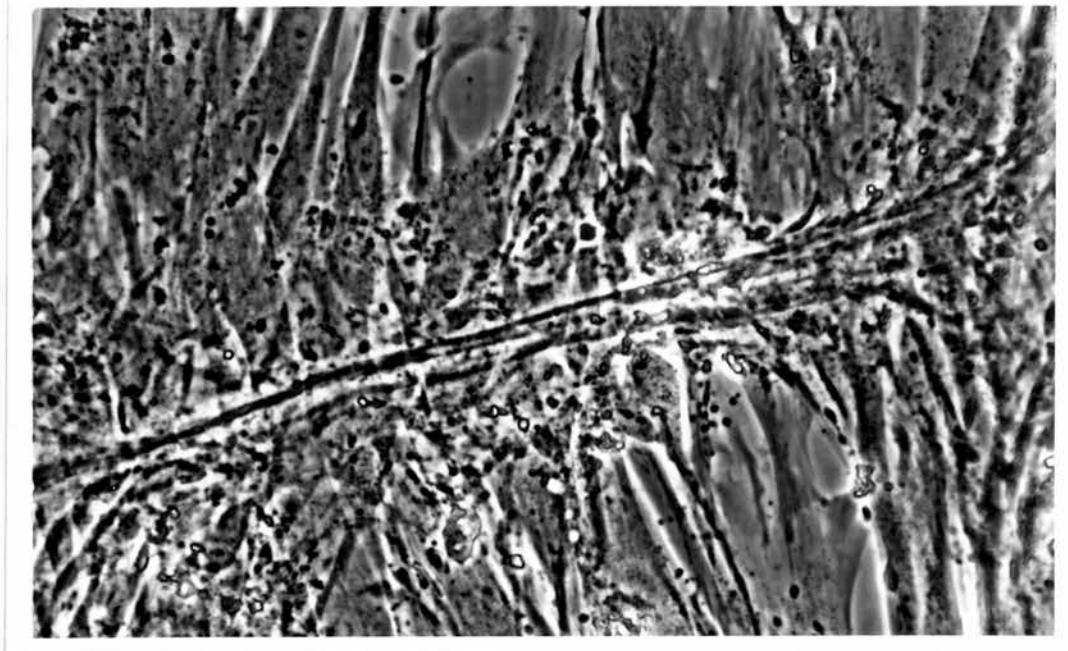


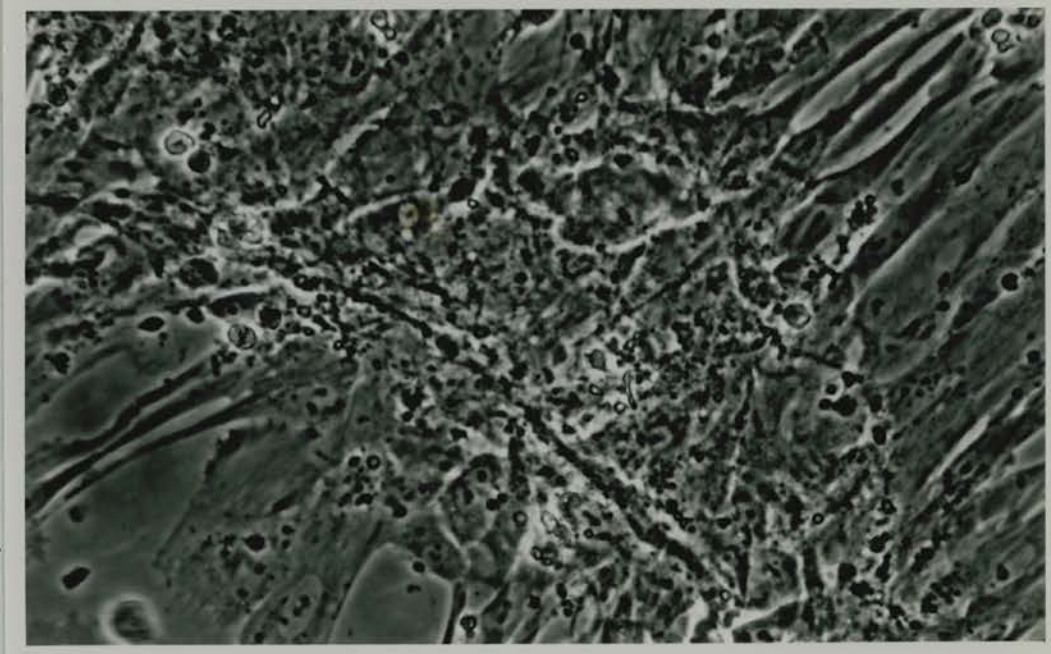
Fig. 88.

TC 80. Anaplastic carcinoma. Grade II elastosis. Myofibroblasts contracted into a cellular ridge with associated fibrillar material.

Fig. 89.

TC 80. Anaplastic carcinoma. Grade II elastosis. Histological section of an aggregation of myofibroblasts stained to display the presence of elastin. There were small but significant quantities of Gomori positive (purple) material.

TC 80 35 days culture.



One such collection of cells from a highly elastotic tumour (TC 80) was formalin fixed, wax embedded and sectioned. Specific staining for elastin, by the Gomori aldehyde fuchsin technique, revealed the presence of small but significant quantities of elastin within the aggregations of cells.

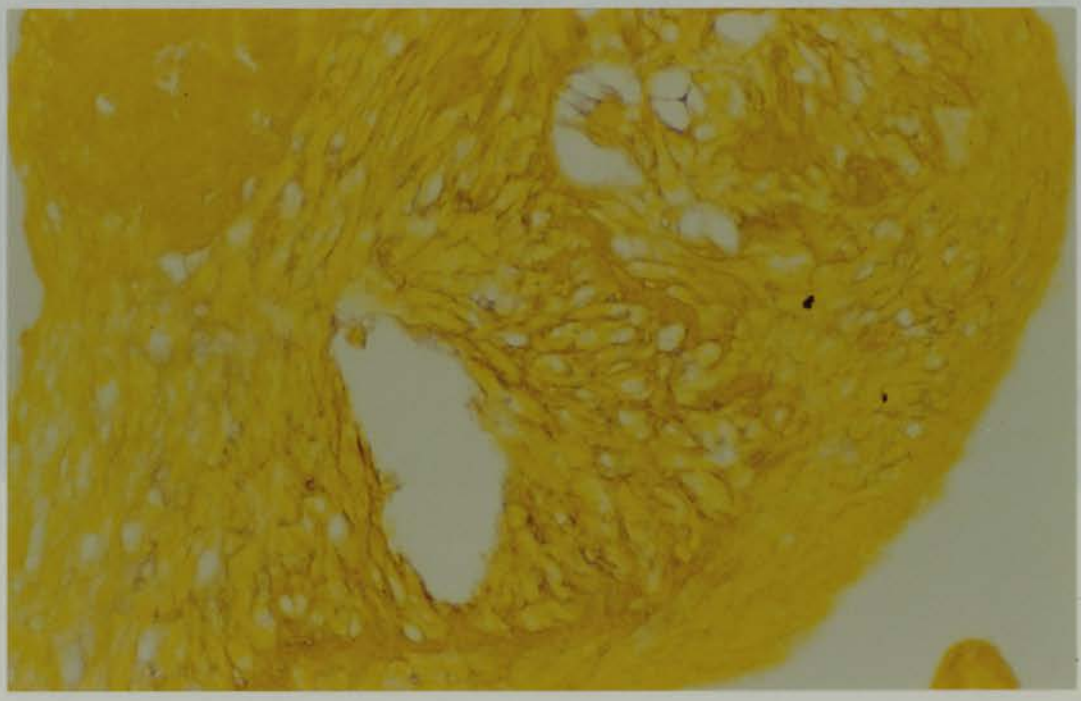
TC 80. 58 days culture. Gomori aldehyde fuchsin/tartrazine.

Original magnification X 72

Fig. 90.

TC 80. 58 days culture. Gomori aldehyde fuchsin/tartrazine

Original magnification X 72



The myofibroblast has been described 'in situ' in carcinoma of the breast before [118, 119, 149] and cells which resemble myofibroblasts have been linked with elastin, collagen and matrix production in arterial walls [178]. It thus seems probable that the cells cultured were, indeed, myofibroblasts. Further proof of the nature of the cells cultured was furnished by transmission electron microscopy.

Fig. 91.

Transmission electron micrograph of a 'fibroblast-like' cell from cultures of TC 76, a highly elastotic anaplastic carcinoma. 31 days culture. The figure illustrates the deeply notched nucleus, multiple nucleoli, microfibrils and glycogen deposits which were present in cells of this type.

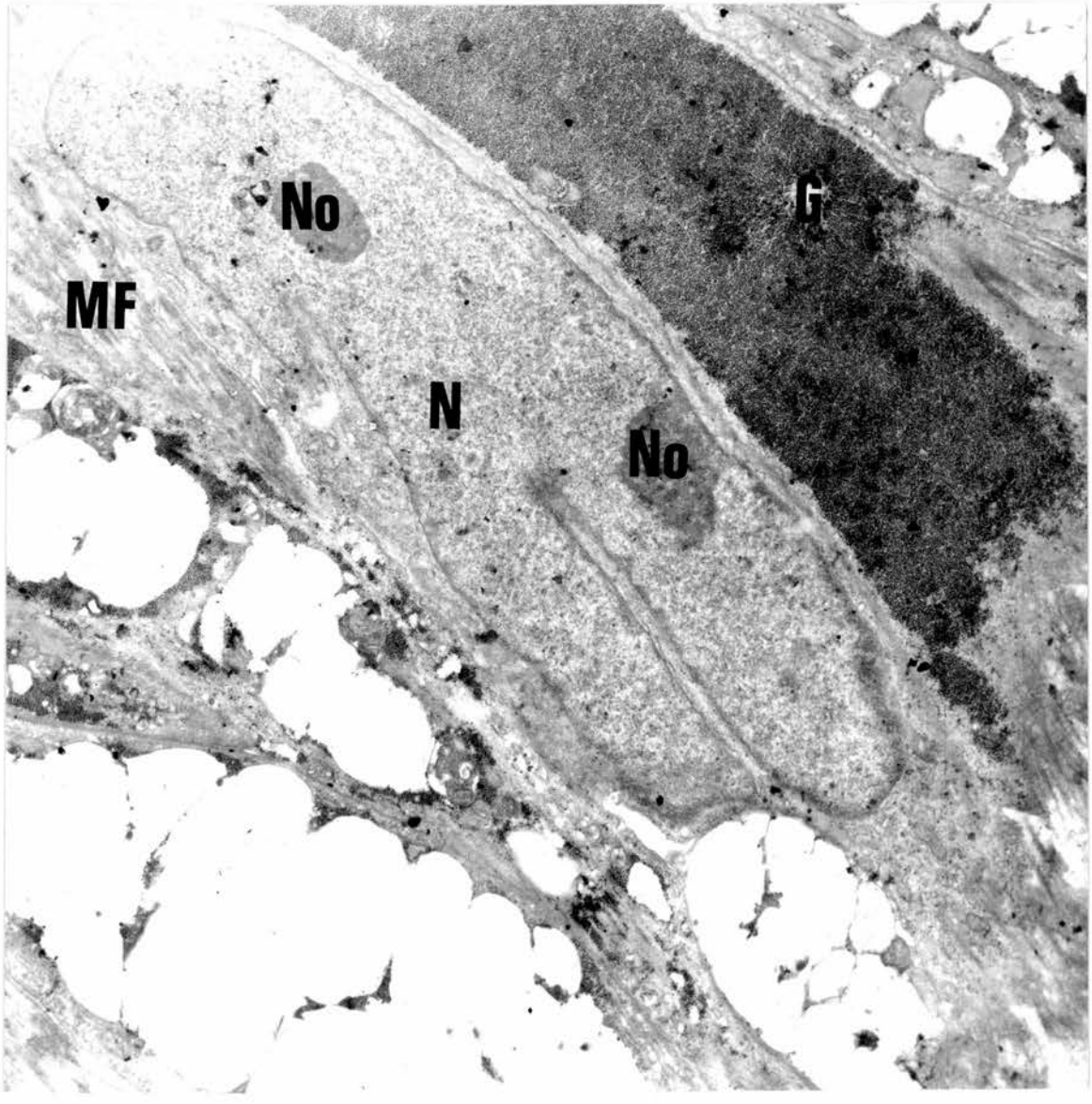
vi) Transmission Electron Microscopy.

Cells of two morphological types which were cultured from TC 76, a highly elastotic anaplastic mammary carcinoma, were examined using the Transmission electron microscope, 31 days after culture was initiated.

a) "Fibroblast-like" cells.

The "fibroblast-like" cells which grew in culture and which were shown, on histological processing, to be capable of elastin production, shared many ultrastructural features with myofibroblasts, as described by Seemayer et al[149].

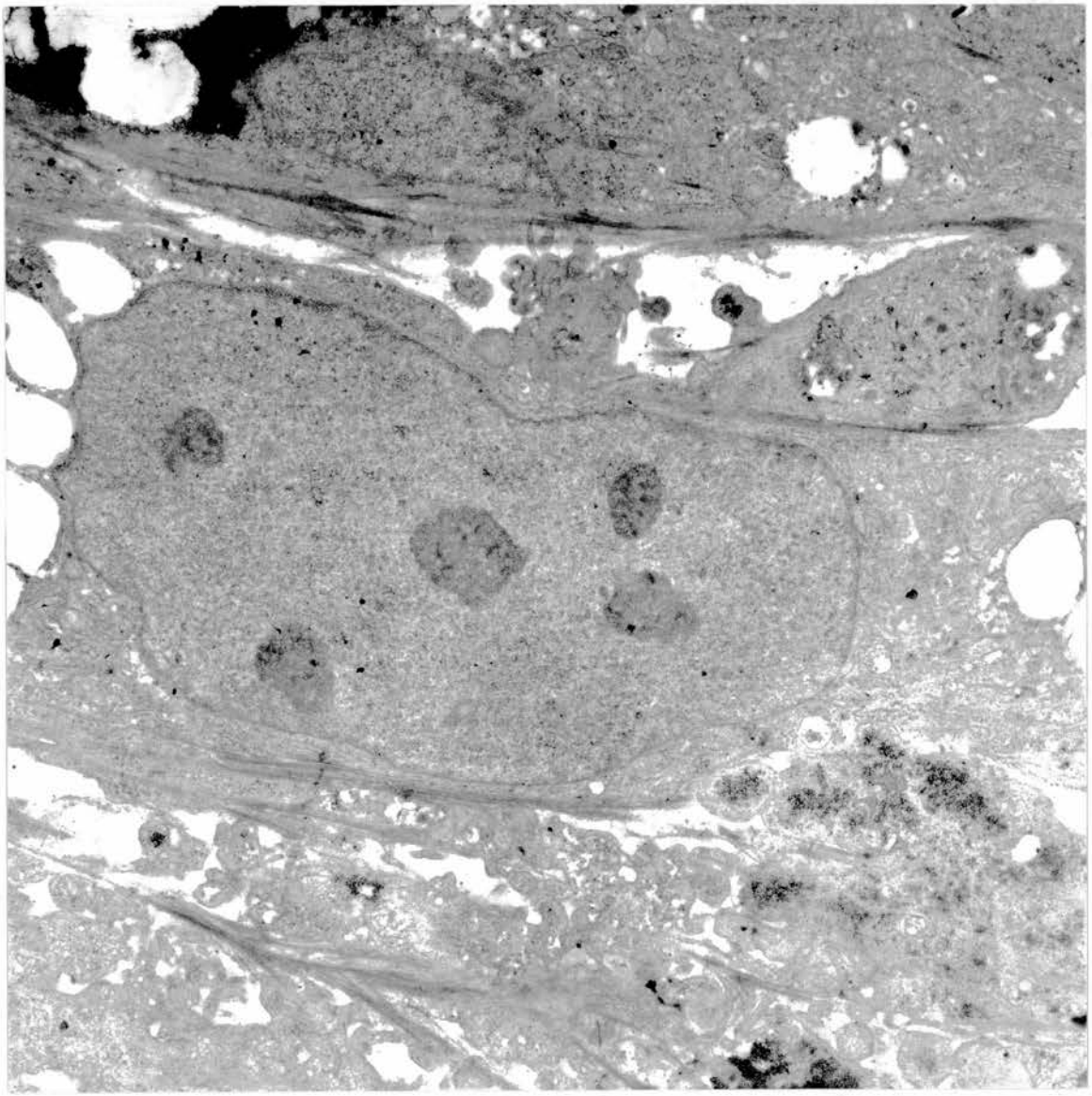
The cells were spindle shaped and contained within them elongated, often deeply notched nuclei with one or two quite prominent electron dense nucleoli.



N - Nucleus No - Nucleolus G - Glycogen MF - Microfibrils. X 6,250

Fig. 92.

Occasionally there were as many as 5 nucleoli present, a possible indication of a rapid growth rate.[45]

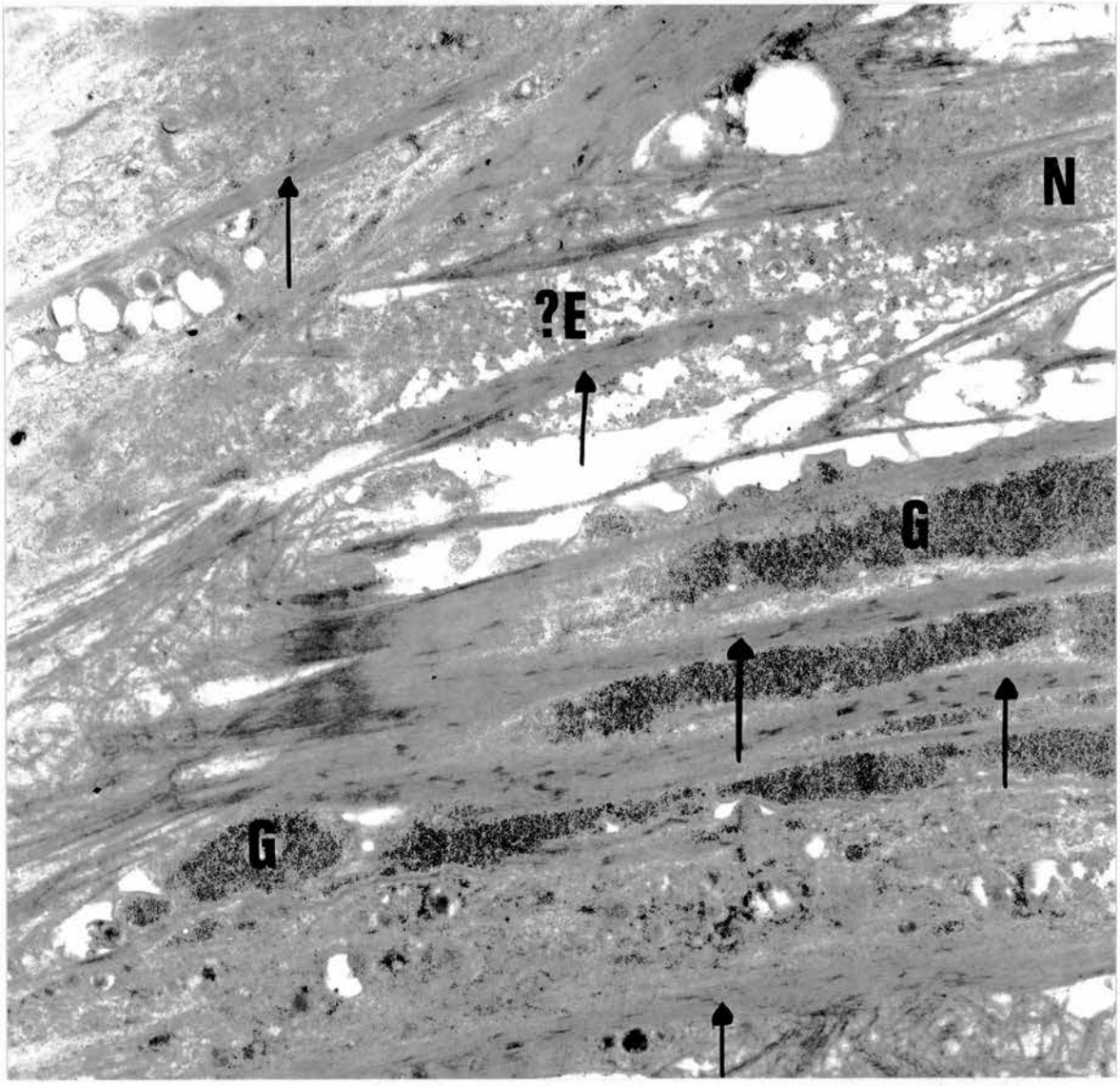


X 6,250

One of the ultrastructural features most characteristic of the myofibroblast is the presence within the cytoplasm of microfibrils - 50 - 70 Å in diameter, concentrated at intervals to form electron-dense 'dense bodies'. This type of structure was a prominent feature of the fibroblast-like cells cultured. Large deposits of glycogen were found/

Fig. 93.
TC 76. Highly elastotic anaplastic carcinoma. 31 days culture. TEM of 'fibroblast-like' cells illustrating the microfibrils, glycogen deposits and the possible location of elastin.

found in all the fibroblast-like cells examined. The presence of glycogen in mammary carcinoma cells has been noted before[124]. In the present study the deposition of glycogen may have been a result of the length of the culture period, representing storage products accumulated within the cells during that time.

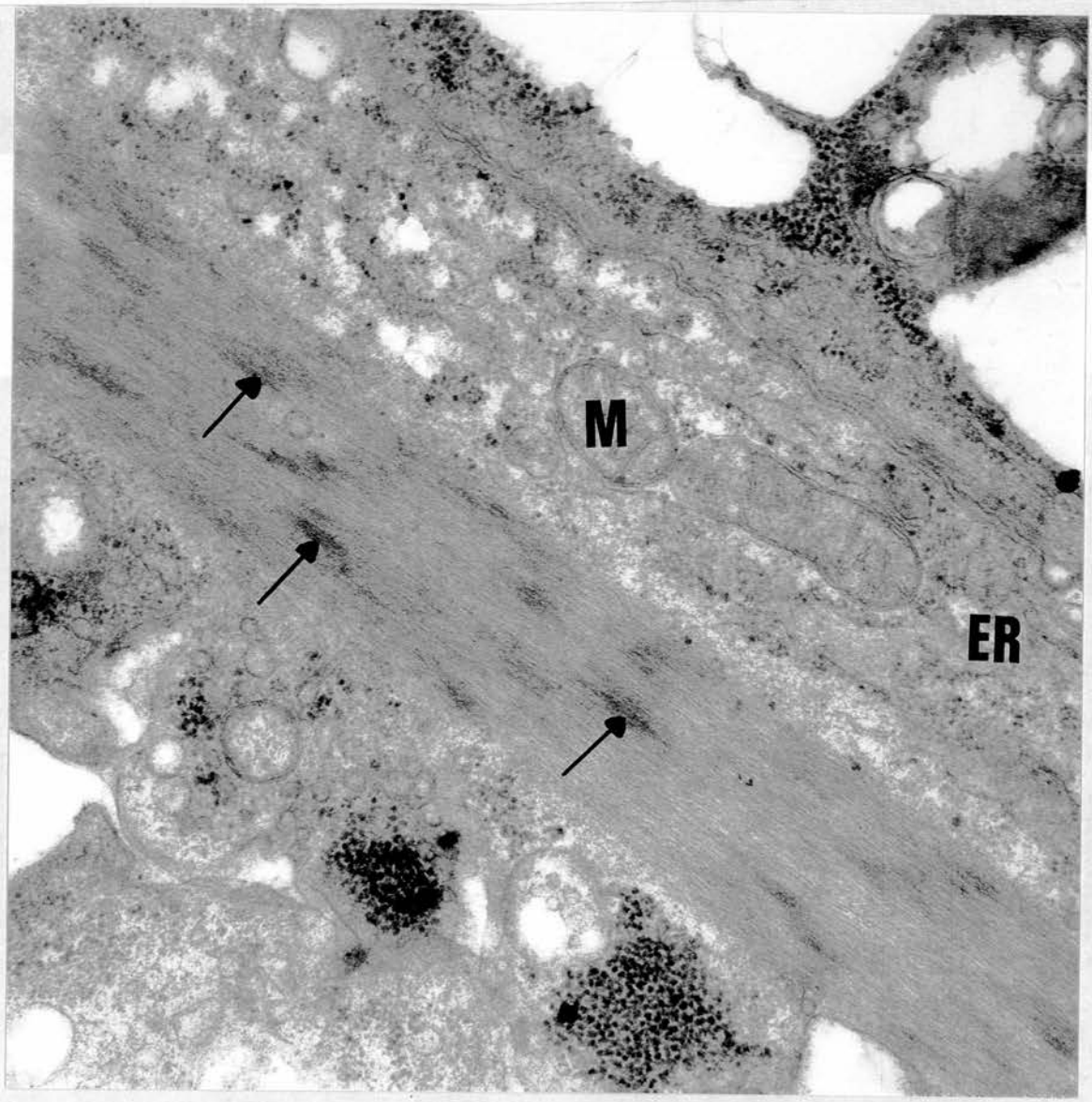


N - Nucleus G - Glycogen ⟶ Microfibrils.

? E - Possible location of elastin deposits in primitive form.

Fig. 94.

TC 76. 31 days culture. Detailed view of microfilaments with dense bodies, mitochondria, endoplasmic reticulum and glycogen deposits found in the 'fibroblast-like' cells.



X 37,500 M - Mitochondrion ER - Endoplasmic reticulum.

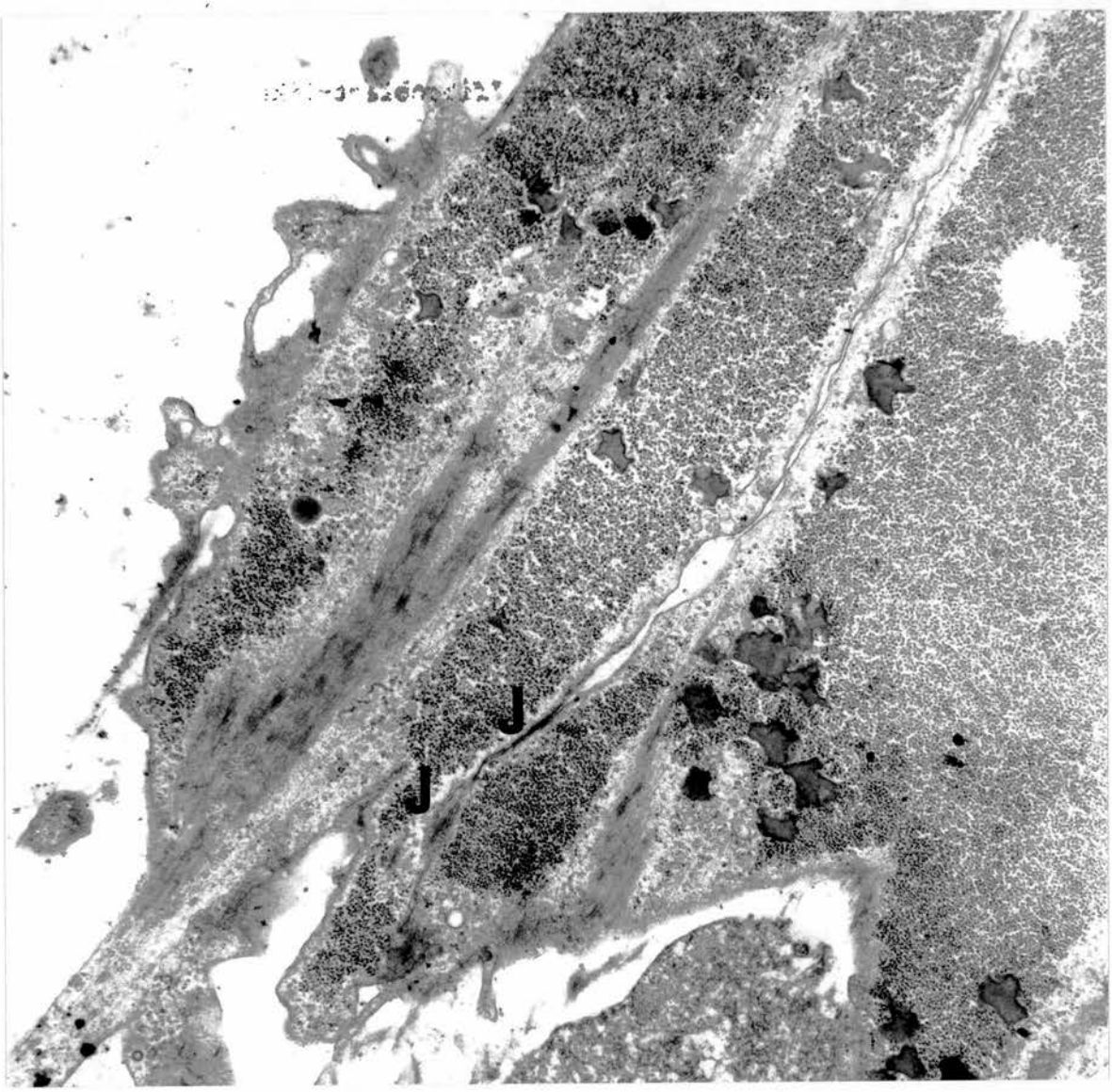
→ Dense bodies.

Fig. 95.

Showing cell-to-cell attachment sites between 'fibroblast-like' cells.

Other features characteristic of myofibroblasts are the presence of pinocytotic vesicles at the cell surfaces and cell-to-cell attachment sites which do not show the typical structure of the epithelial cell desmosome.

Pinocytotic vesicles were not located but there were a few cell-to-cell junctions.

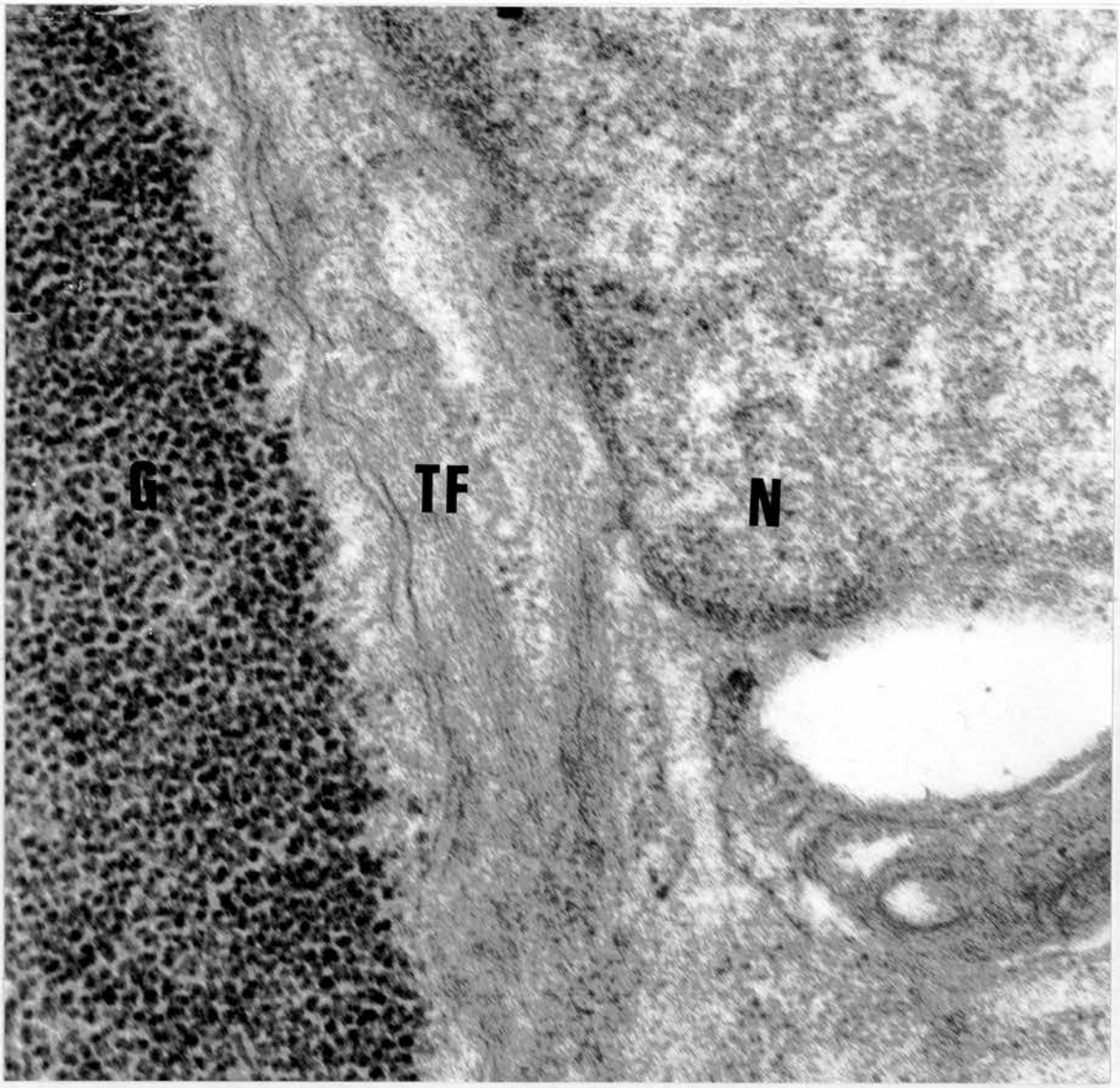


J - Intercellular junction X 15,000

The endoplasmic reticulum in these cells was not very well developed as compared with that depicted in cells designated myofibroblasts by other workers[118, 119, 149]. The lack of rough surfaced ER may have been a reflection of the age of the cell cultures (31 days), indicating that the cells were approaching senescence. Another possibility, however, is that the cells were not typical myofibroblasts but myoepithelial cells or a cell type intermediate between the two. Myoepithelial cells share many ultrastructural features with myofibroblasts[114, 119] The cells are fusiform, have indentate nuclei and prominent nucleoli, microfilaments with dense bodies in the cytoplasm and pinocytotic vesicles. Myoepithelial cells 'in vivo' have true desmosomes between the cells and also produce a continuous basal lamina. No basal lamina whatever was detected in the cell cultures examined and no true desmosomes were located, lending support to the identification of the cells as myofibroblasts. There was, however, some evidence of the presence of tonofibrils in the cytoplasm - a feature of myoepithelial cells. To date there are no reports of the growth of myofibroblasts in tissue culture and it may be that the artificial conditions in which the cells are grown confer unique characteristics upon them.

Fig. 96.

TC 76. 31 days culture. Tonofilaments within 'fibroblast-like' cells.



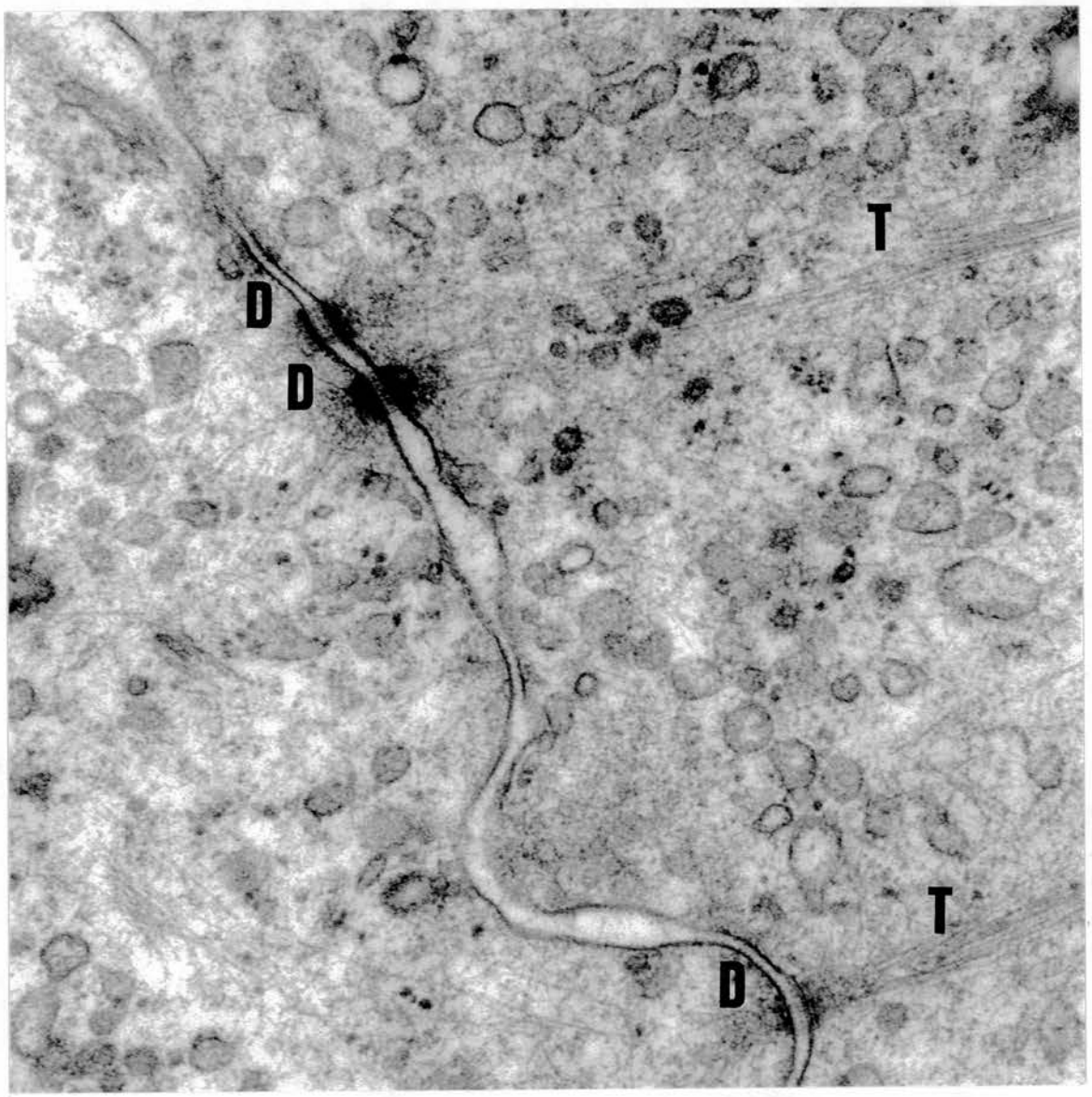
X 62,500 G - Glycogen N - Nucleus TF - Tonofibrils.

Fig. 97.

TC 76. Highly elastotic anaplastic carcinoma. 31 days culture. Desmosomes with associated tonofilaments found between 'epithelial-like' cells in these cultures.

b) "Epithelial-like" cells.

The "epithelial-like" cells from cultures of TC 76 which were examined with the transmission electron microscope were shown to be true epithelial cells by the presence of classical intercellular desmosomes.



X 62,500 D - Desmosome. T - Tonofilaments.

Other, intracellular, structures were suggestive of the apocrine variant of the mammary cell and subsequent review of the diagnostic biopsy (see Section III p. 143) confirmed that cells of apocrine type were present within the tumour.

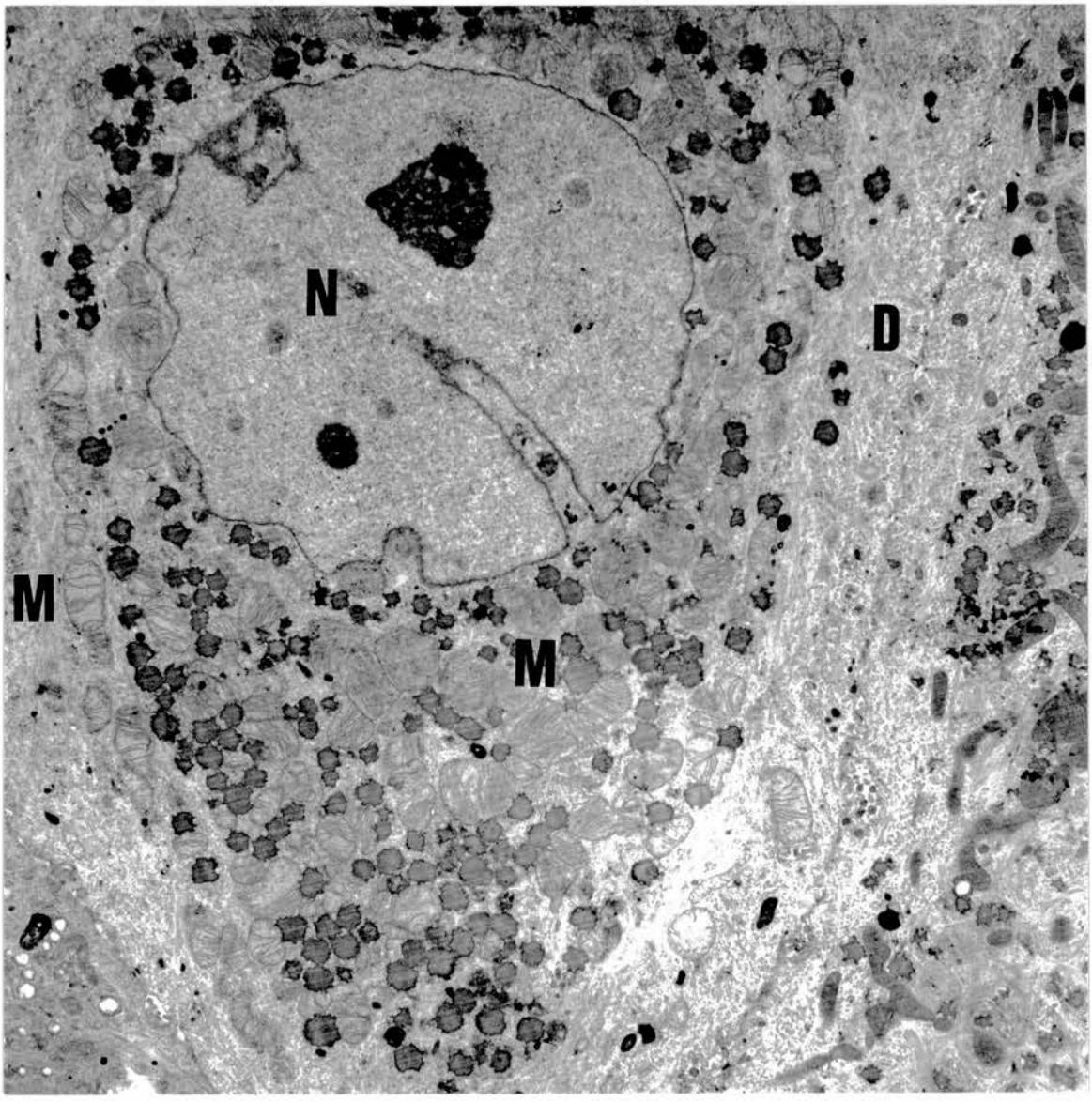
Fig. 98.

View of 'epithelial-like' cells in cultures of TC 76, a highly elastotic anaplastic carcinoma, 31 days culture, showing features characteristic of apocrine cells.

'In vivo' one of the most characteristic features of apocrine cells is an increase in the number of mitochondria [3, 115] . Their size and shape is very variable and the cristae are long, thin, few in number and often incomplete. The mitochondria are also relatively electron dense.

Other features taken to be representative of apocrine cells include prominent rough endoplasmic reticulum, bundles of cytoplasmic filaments resembling keratin among the mitochondria and the presence of short microvilli at the apical plasma membrane.

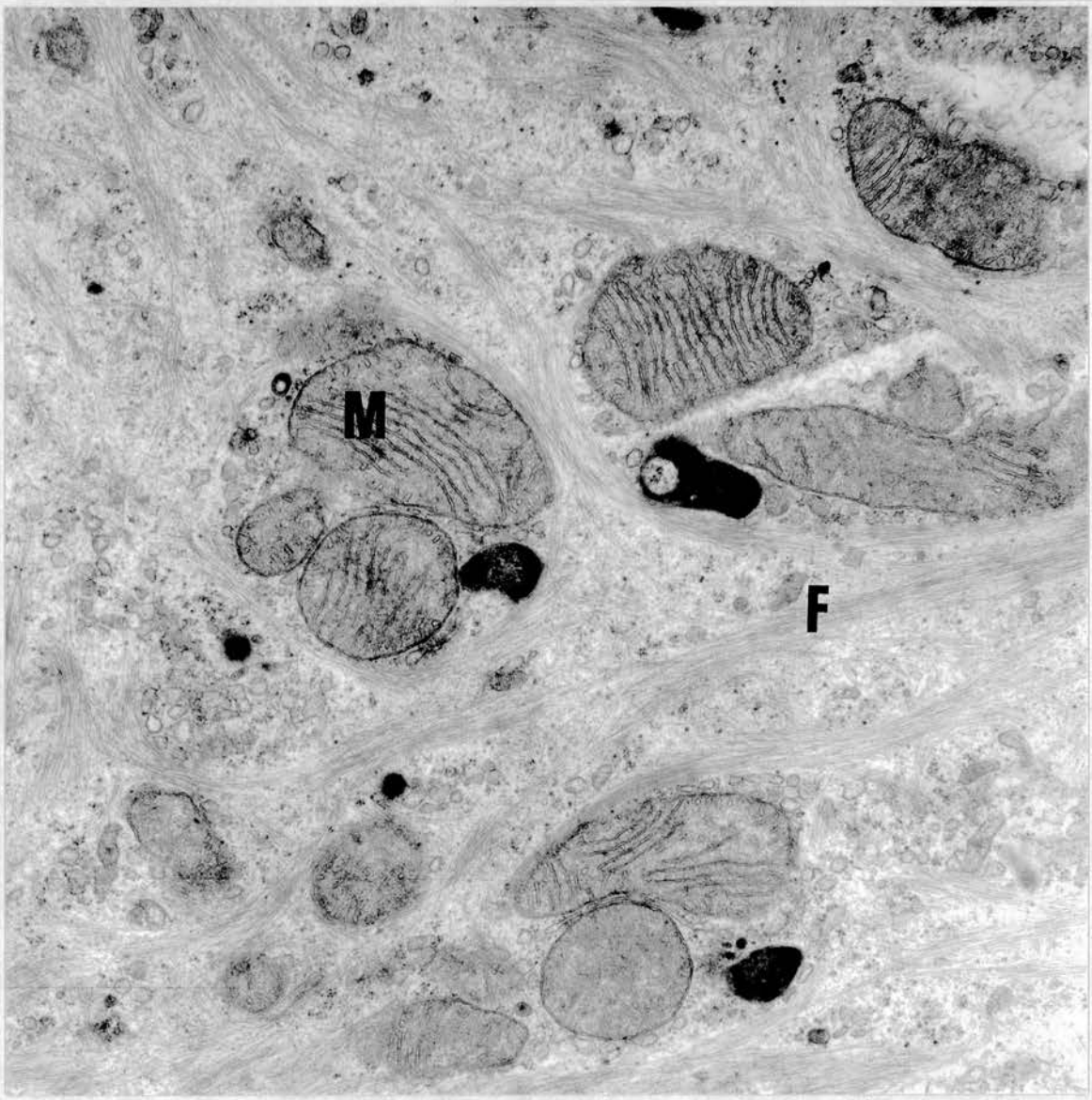
All these features were clearly apparent in the cells cultured.



X 3,750 N - Nucleus D - Desmosome M - Mitochondrion.

Fig. 99.

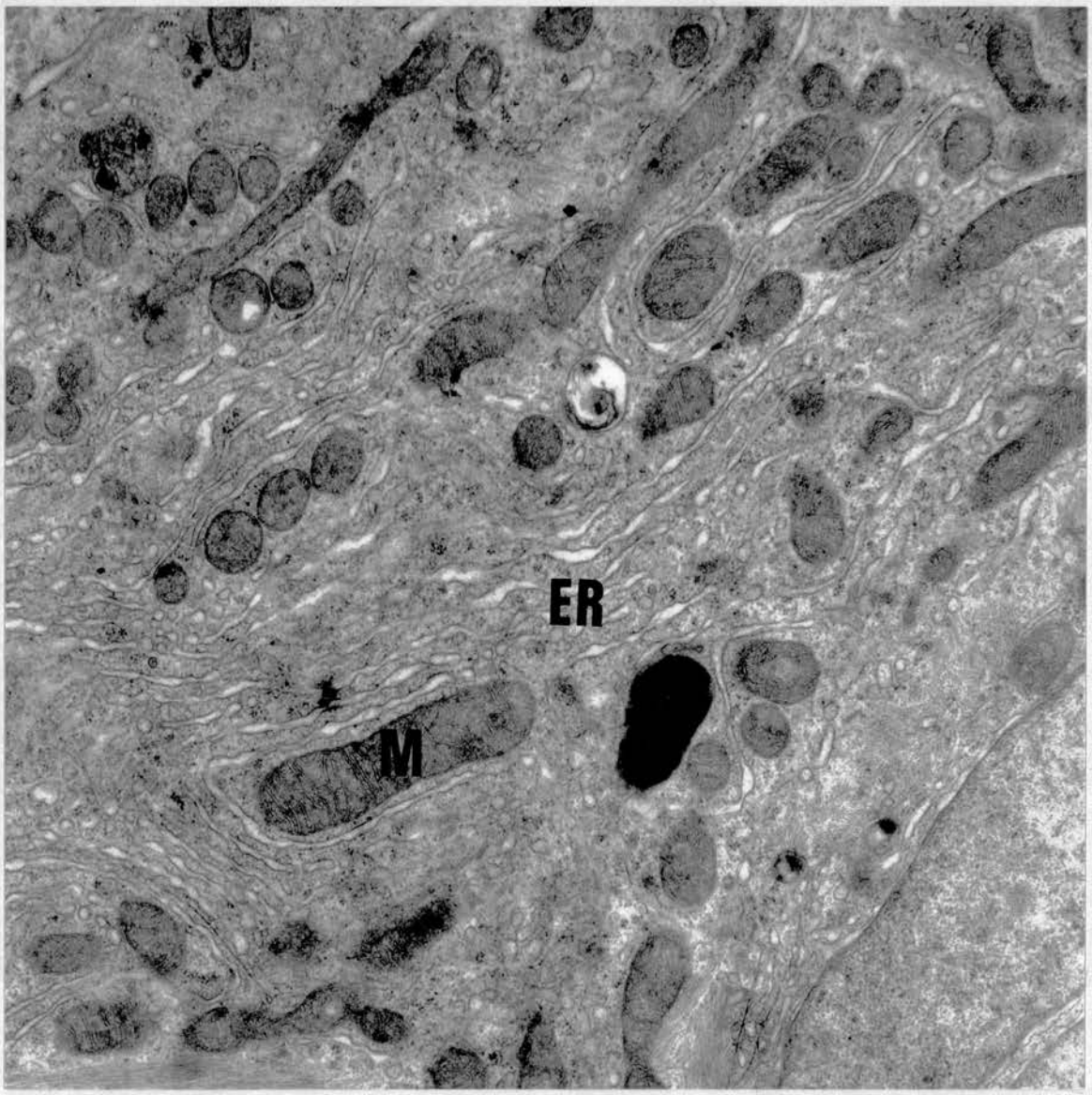
TC 76. 31 days culture. Detailed view of apocrine-type mitochondria with sparse, often incomplete cristae and cytoplasmic filaments.



X 25,000 M - Mitochondrion F - Cytoplasmic filaments.

Fig. 100.

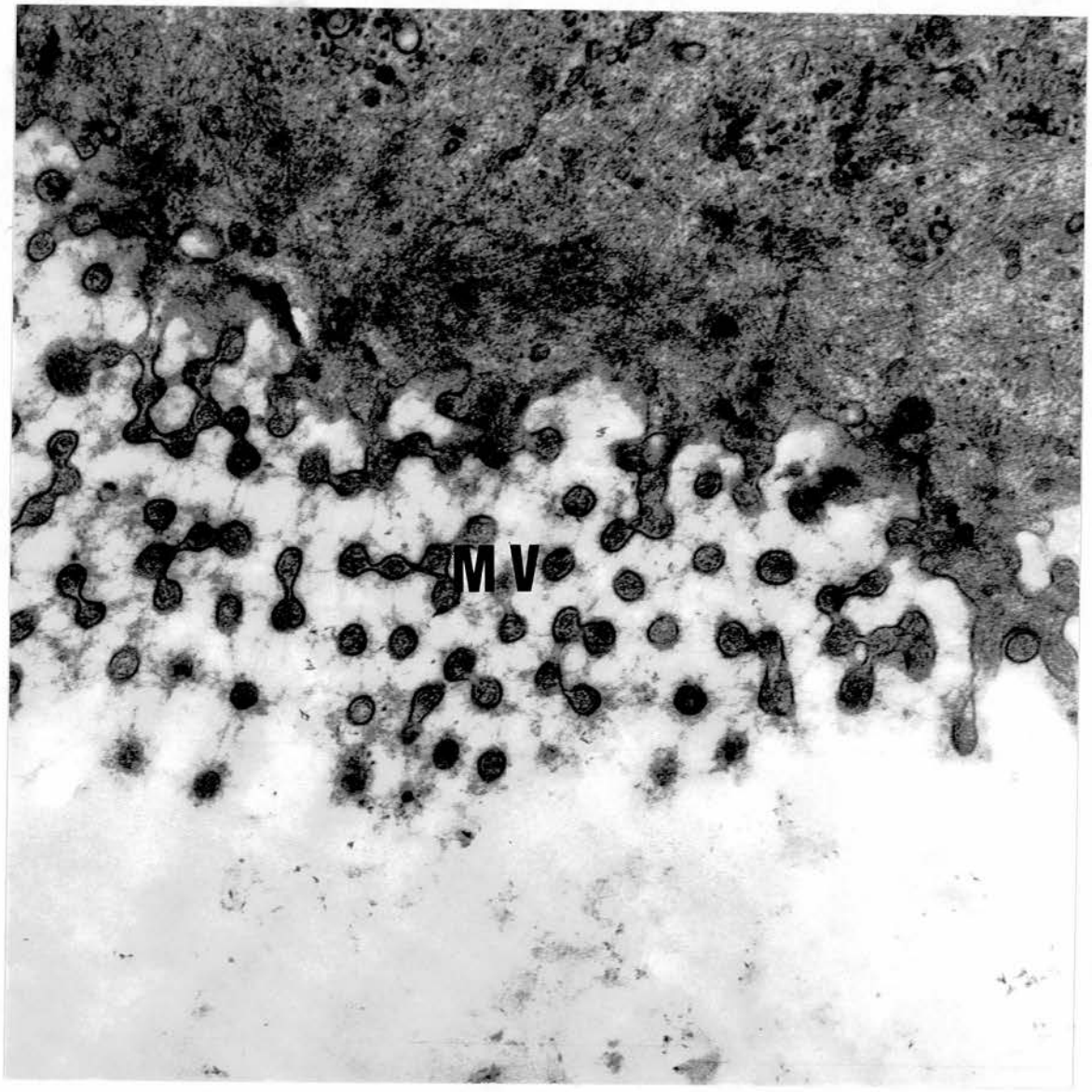
TC 76. 31 days culture.



X 25,000 M - Mitochondrion ER - Endoplasmic reticulum

Fig. 101.

Cross section through short terminal microvilli. X 37,500.



MV - Microvilli.

Extensive infolding of the plasma membrane, a further characteristic of apocrine cells [3, 115], was not seen. This feature of the apocrine cell may only be apparent 'in situ' and fail to develop in culture.

There was no ultrastructural evidence to suggest the formation of elastin within these cells which, earlier in their culture history, had been shown to "bleb" material identified histologically as elastin.

However, the cultures were not examined with the transmission electron microscope until 31 days after culture initiation and, owing to the apparently phasic nature of the "blebbing" phenomenon, all elastin may have been released from the cells.

Fig. 102.

TC 33. Peripheral spindling at the edges of epithelial cell colonies.

Phase contrast. Original magnification x 150

Fig. 103.

TC 33. Peripheral spindling at the edges of epithelial cell colonies.

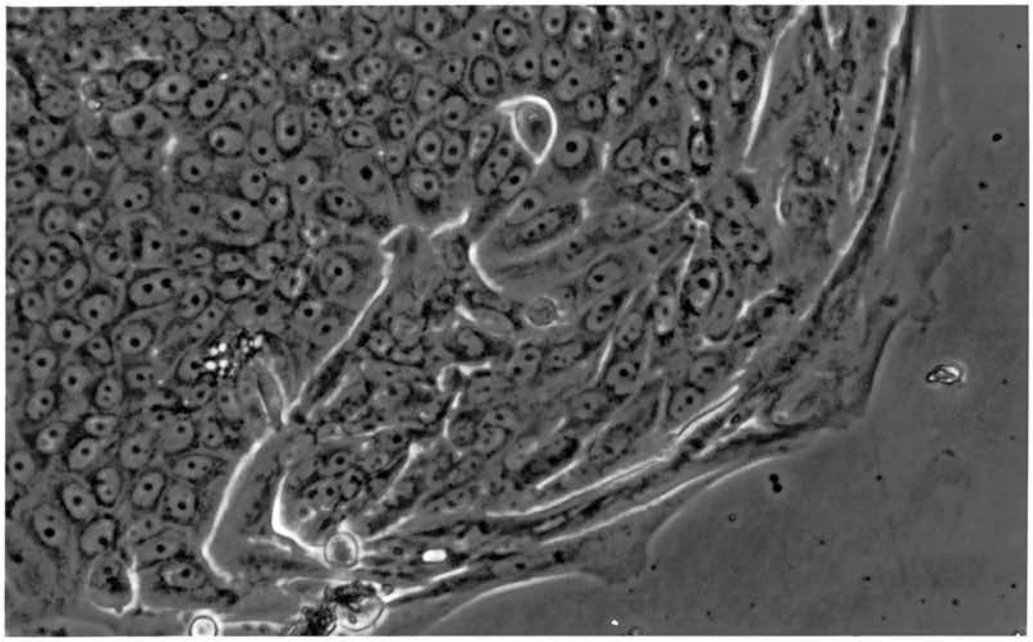
Phase contrast. Original magnification x 150

SECTION II.

PERIPHERAL SPINDLING AND "HALO" FORMATION

A common observation in all the breast tissue cultures was the "peripheral spindling" of epithelial cells. Cells which had originally grown as typically polygonal epithelial cells elongated to take on a fibroblast-like morphology.

TC 33. Anaplastic, small cell carcinoma. Moderate 'stromal reaction', moderate elastosis. 12 days culture.



TC 65. Anaplastic carcinoma. Grade II elastosis. 8 days culture.

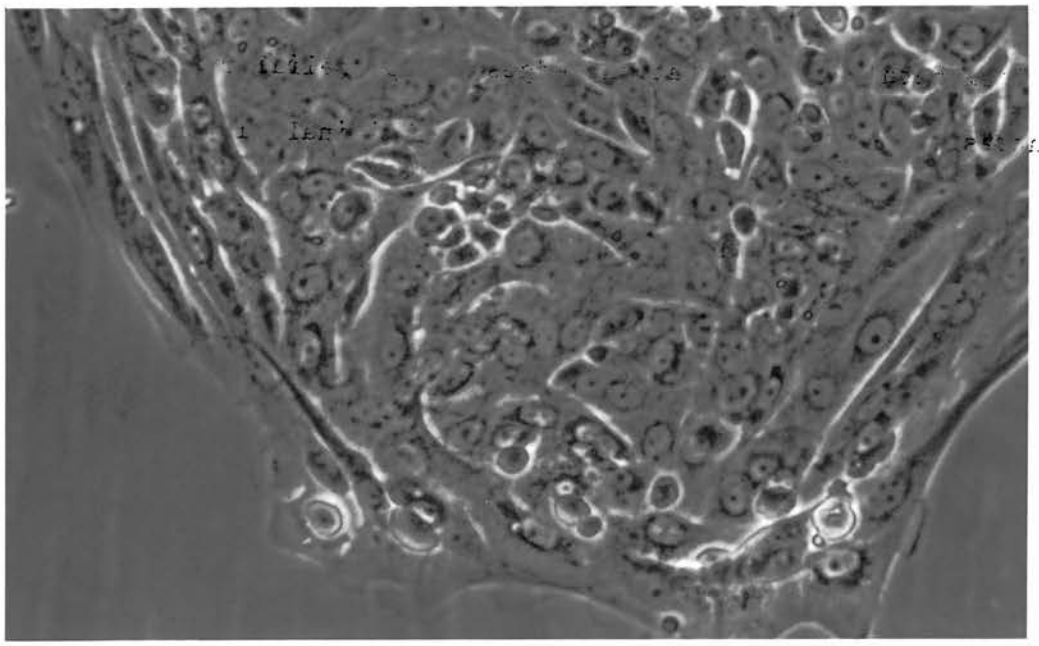


Fig. 104.

TC 23. Refractile, spindled cells at the periphery of an epithelial cell sheet. Phase contrast. Original magnification x 150

As mentioned in the survey of the literature, this type of observation has been made by other workers.[47, 163, 164]. Good photographic reproductions of the phenomenon appear in a paper by Ebner et al[42] (1961) describing the growth of bovine mammary tissue in culture. Hallowes et al[73] and Gaffney and Pigott[57], in their papers describing two types of epithelial cell, E and E[1], which they grew from human mammary carcinoma both published pictures of epithelial 'E' cell islands with peripherally spindled cells.

In most instances in the present study, when viewed with phase contrast microscopy, the edges of the elongated cells and sometimes also the polygonal cells were highly refractile.

TC 23. Anaplastic carcinoma, partly intraduct.
12 days culture.

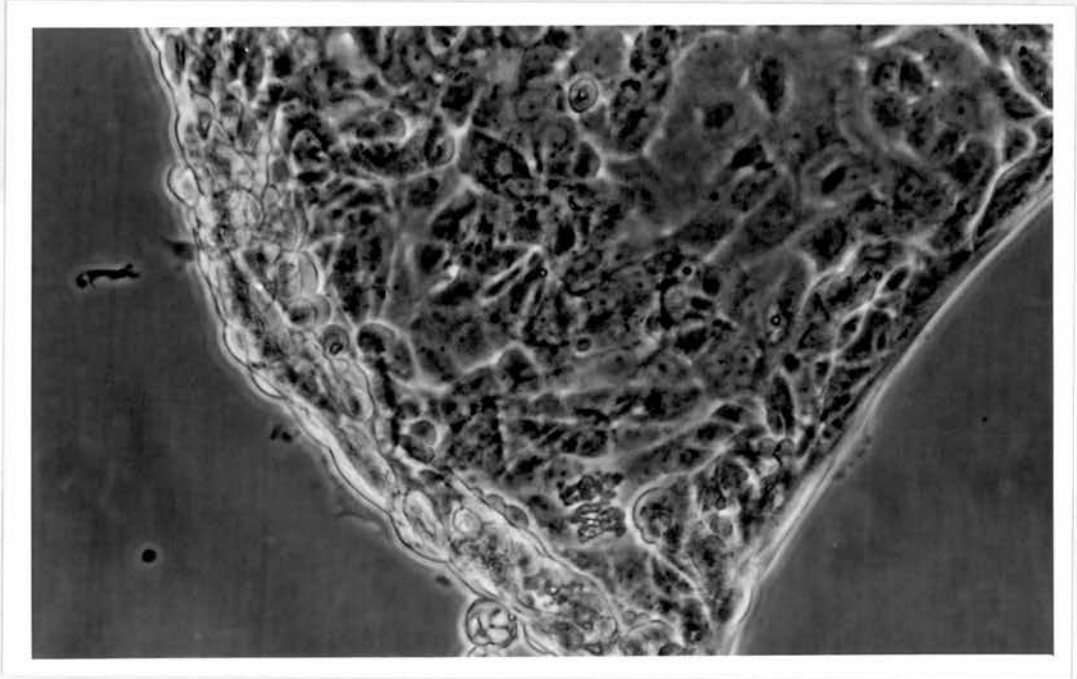


Fig. 105.

TC 9. Refractile areas around each individual polygonal epithelial cell.

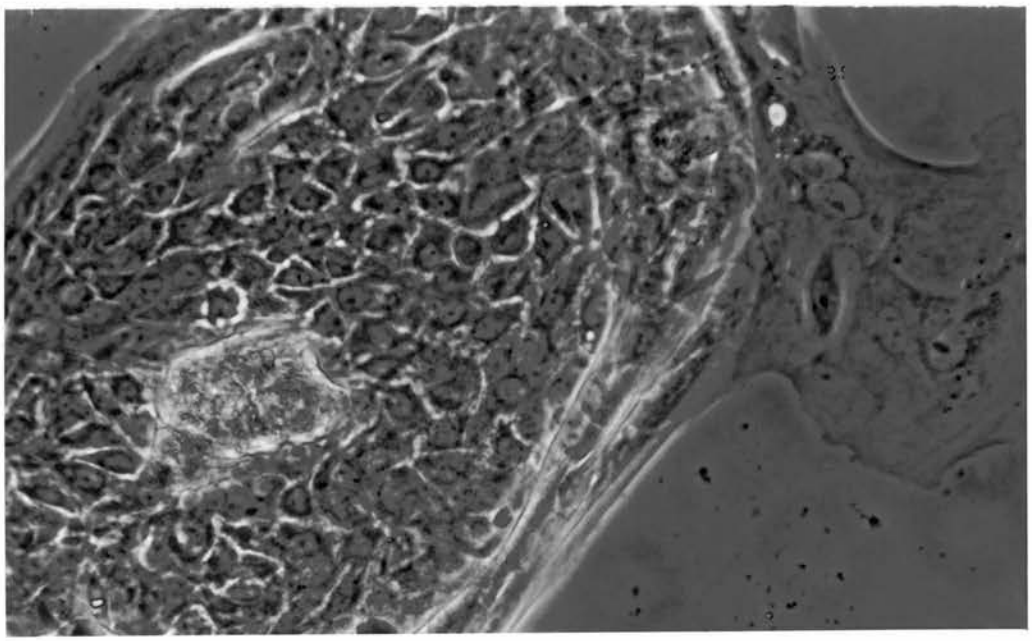
Phase contrast. Original magnification x 150

Fig. 106.

TC 20. A detached, floating group of highly refractile epithelial cells.

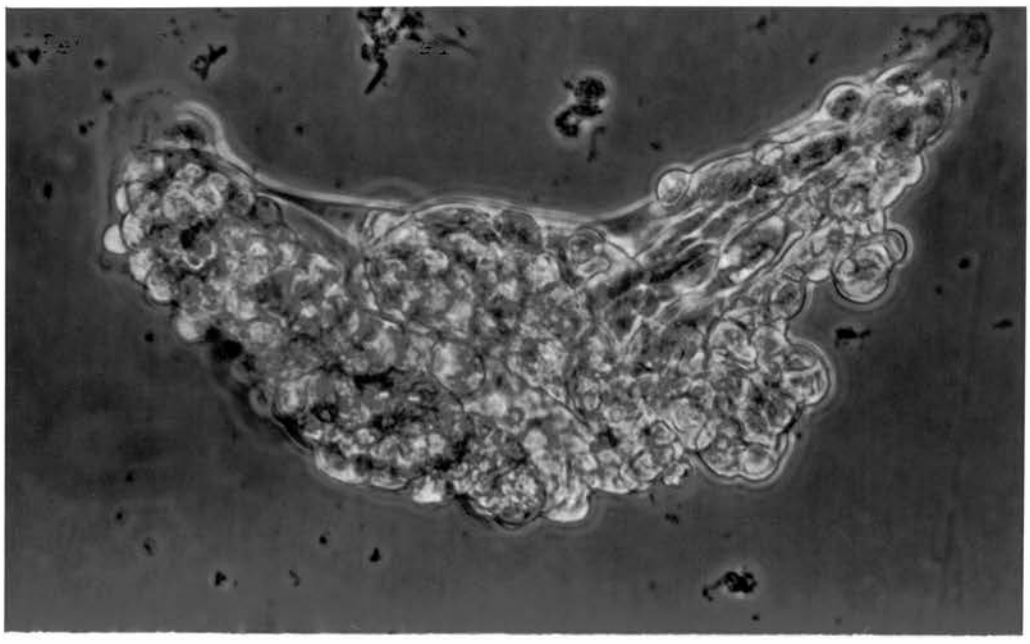
Phase contrast. Original magnification x 150

TC 9. Fibrocystic disease. 16 days culture.



The development of a refractile 'halo' appeared to presage the cessation of growth and groups of cells eventually detached from the floor of the flask to float in the medium.

TC 20. Scirrhous carcinoma. 13 days culture.



THE DEMONSTRATION OF RETICULIN FORMATION

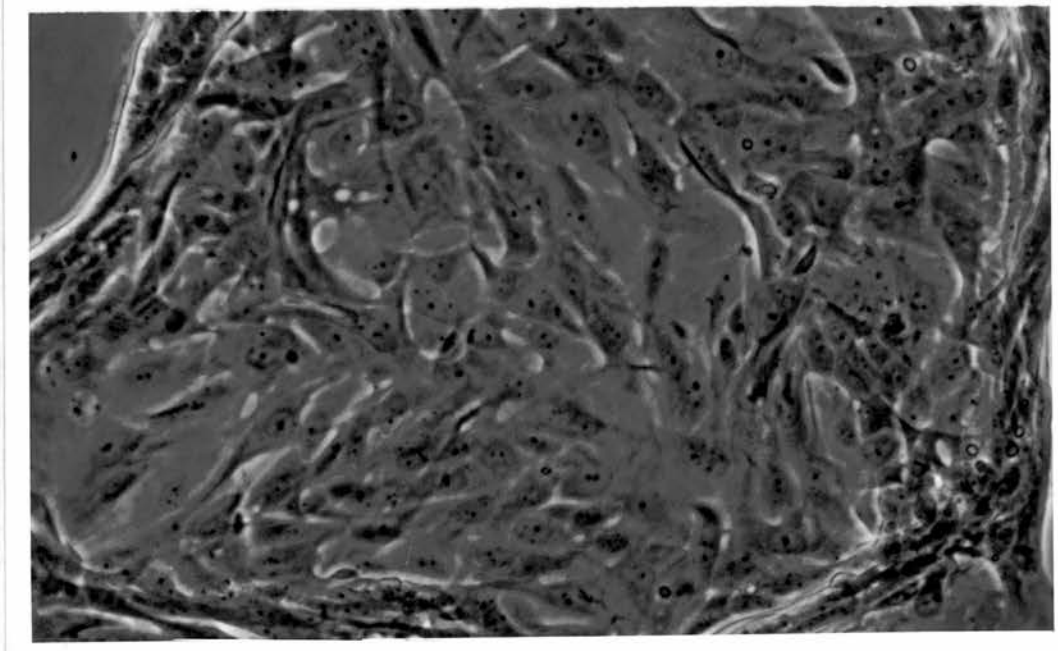
There was a strong indication in many of the cell groups which developed 'haloes' that the cells were bound together by something more than mutual adhesion. In view of the evidence cited, that tumour cells are capable of secreting connective tissue elements, it was considered possible that the material binding the cells together might be collagen.

Initial attempts to demonstrate collagen in cultures such as those illustrated, using Masson's trichrome staining technique[102], were unsuccessful.

Subsequently Gordon and Sweets' silver impregnation method[61] was applied to the cultures and the presence of reticulin was demonstrated in all those areas which, under phase contrast microscopy, had exhibited a 'halo'.

In most cases the reticulin had been laid down at the edge of the cell group, "binding" the edge of the epithelial pavement and apparently preventing expansion.

Fig. 107. TC 42. Anaplastic carcinoma. Variable 'stromal reaction', moderate elastosis. 9 days culture.
Typical group - phase contrast microscopy.
Original magnification x 150



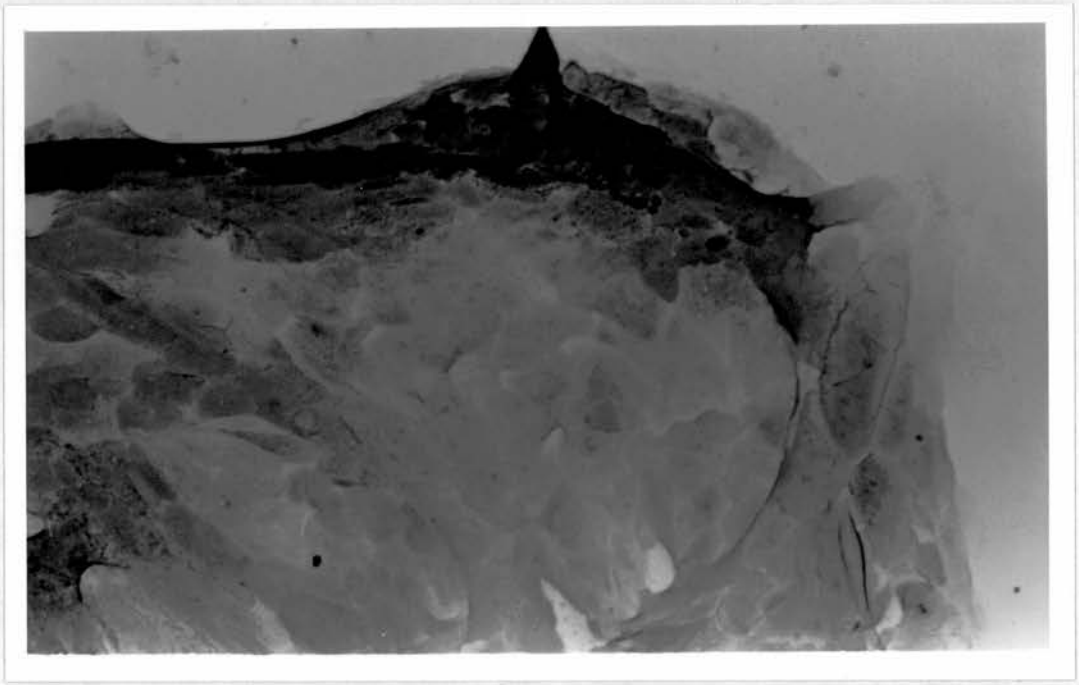
Figs. 108 and 109.

TC 42. Stained preparations of the culture depicted in Fig. 110.

Original magnification x 150

TC 42. Gordon and Sweets' silver impregnation for reticulin.

Note strongly positive peripheral staining.



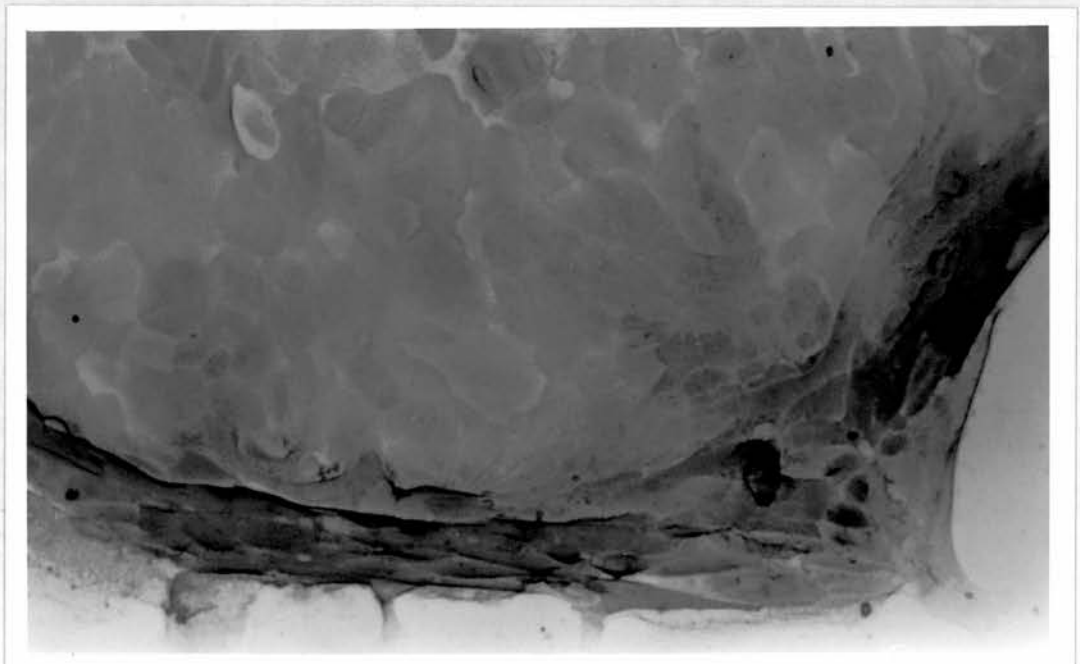


Fig. 110.

TC 70. Gynaecomastia. 9 days culture. Gordon and Sweets' silver impregnation. A 'bound' group of cells. Note positive peripheral staining. Plain light. Original magnification x 150

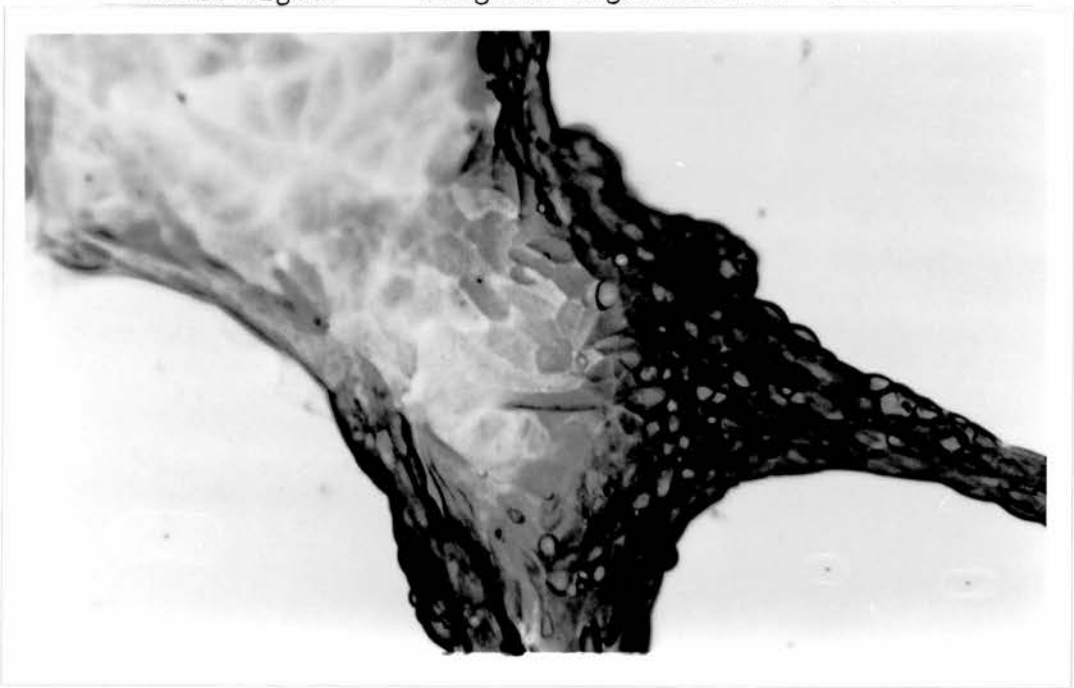
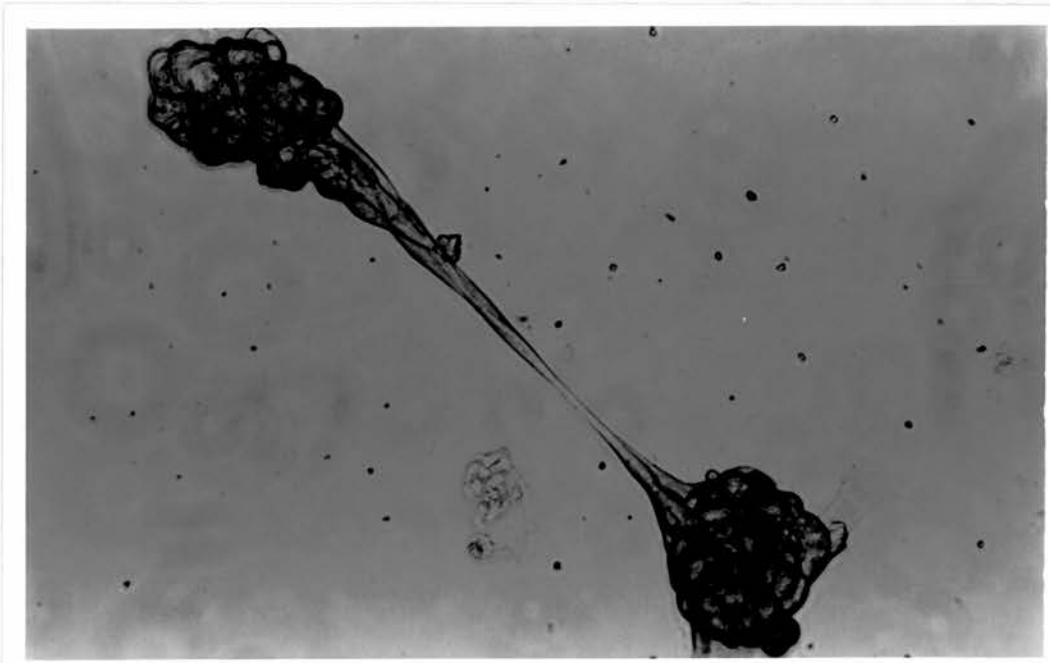


Fig. 111.

TC 6. Fibroadenosis. Gordon and Sweets' silver impregnation. 28 days culture. A 'bound' group of cells. Phase contrast. Original magnification x 150



Often, in very pleomorphic cell pavements, where cells piled up on one another to form several layers, there were groups of cells which appeared to be 'encased' in reticulin.

Fig. 112.
TC 23. Anaplastic carcinoma, partly intraduct. 7 days culture.

Part of typical cell pavement. Note cellular pleomorphism.
Original magnification x 150

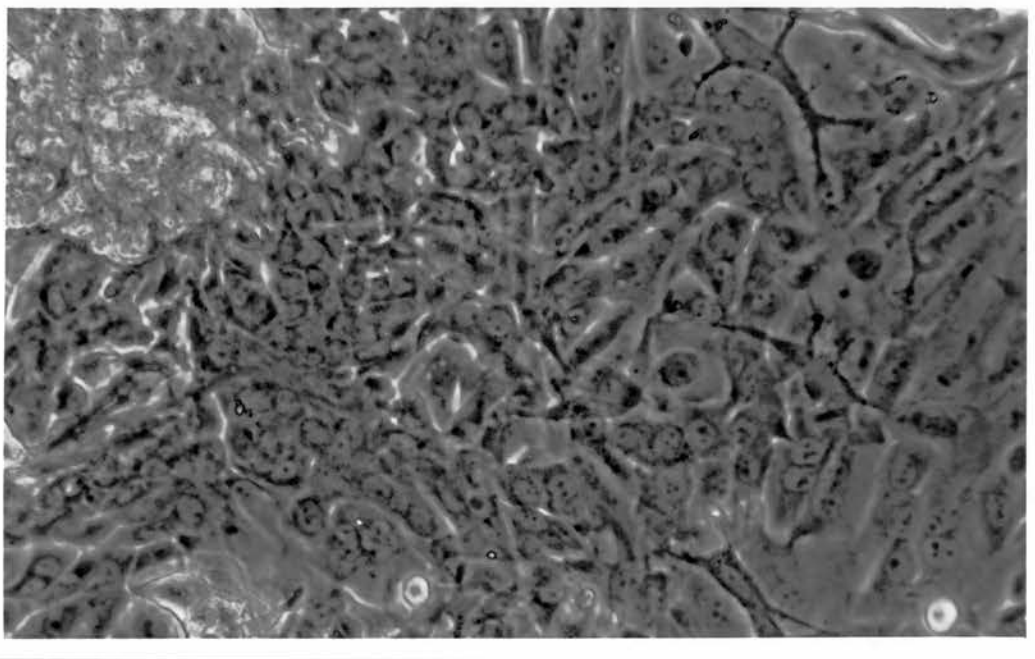


Fig. 113. TC 23. 15 days culture. Gordon and Sweets' silver impregnation method for reticulin. Note uneven distribution of silver deposition.

Original magnification x 150

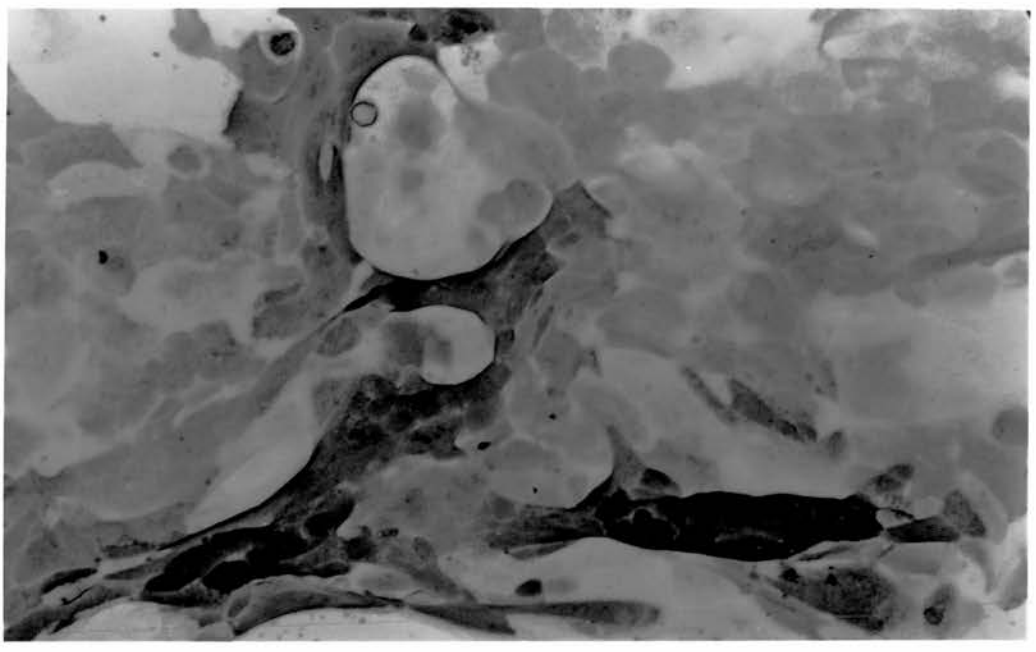


Fig. 115.

Gordon and Sweets' silver impregnation technique applied to the same cell pavement as that depicted in Fig. 114. Plain light.

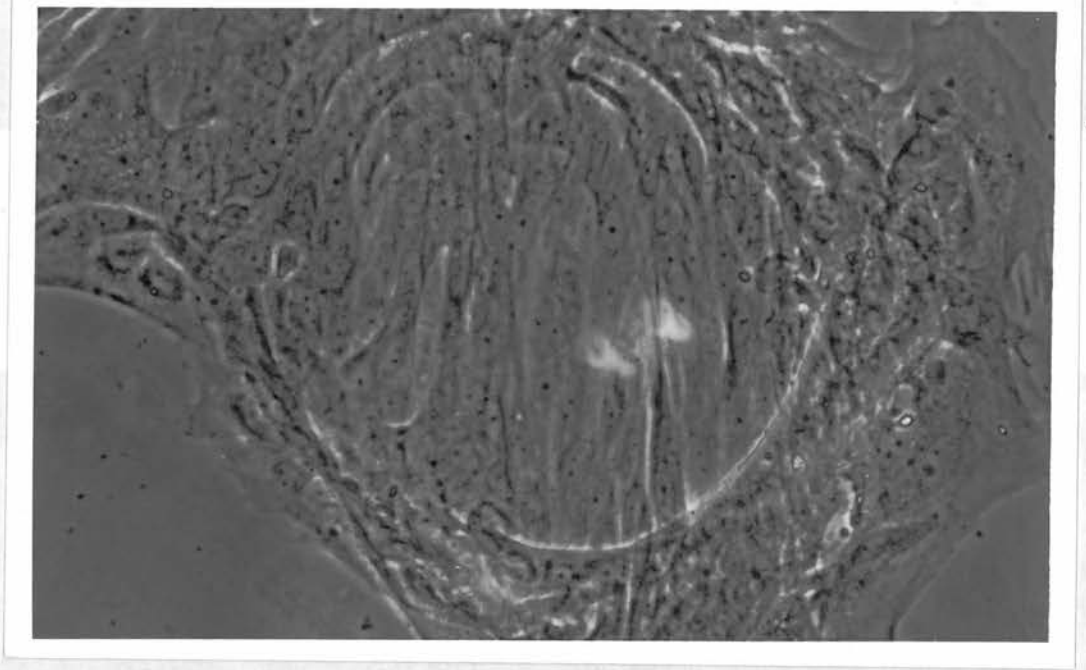
Original magnification x 150

Fig. 114.

TC 41. Anaplastic carcinoma. Variable 'stromal reaction'.

6 days culture. Phase contrast.

Original magnification x 150



Gordon and Sweets' silver impregnation technique.

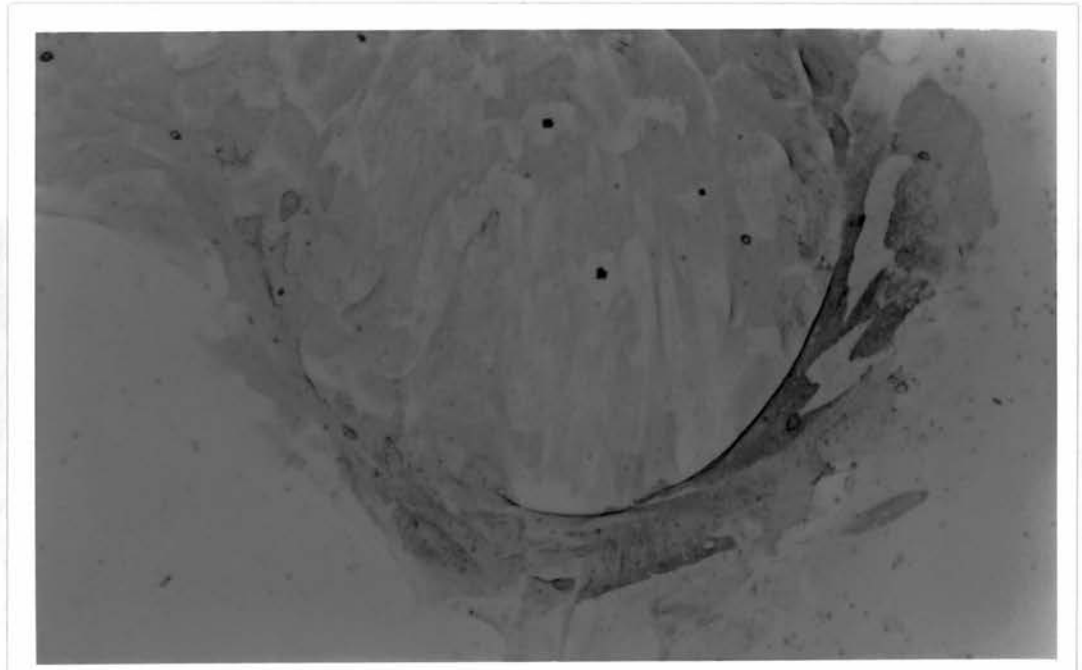


Fig. 116.

TC 33. Cell pavement showing pericellular refringence.

Original magnification x 150

Fig. 117.

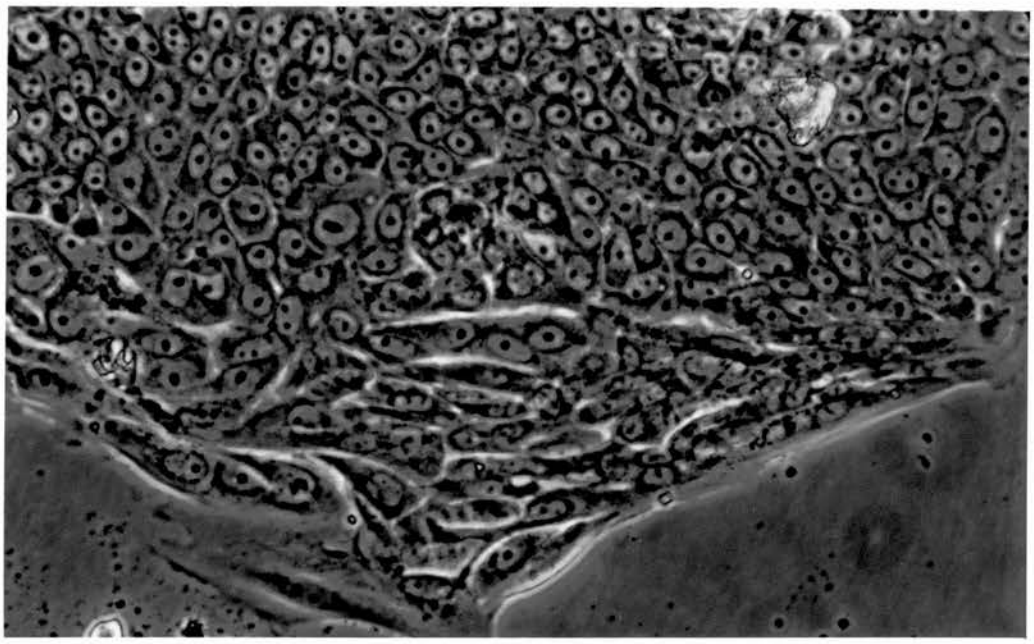
TC 33. An equivalent cell pavement to that depicted in Fig. 116 stained to demonstrate pericellular reticulin deposits. Plain light.

Original magnification x 150

Sometimes there were reticulin deposits around each polygonal epithelial cell.

TC 33. Anaplastic, small cell carcinoma. Moderate 'stromal reaction', moderate elastosis. 12 days culture.

Typical group. Phase contrast.



Gordon and Sweets' silver impregnation for reticulin.

Note peri-cellular reticulin.

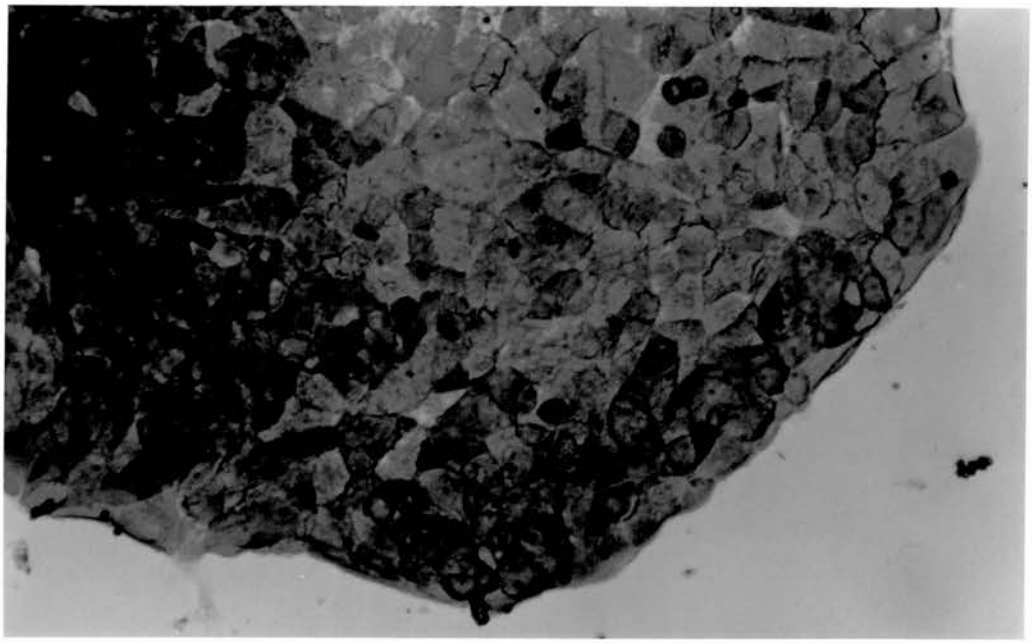


Fig. 118.

TC 33. An equivalent cell pavement to that depicted in Fig. 116 stained to demonstrate pericellular reticulin deposits. Plain light.

Original magnification x 150

TC 33. Gordon and Sweets' silver impregnation for reticulin. Note peri-cellular reticulin.

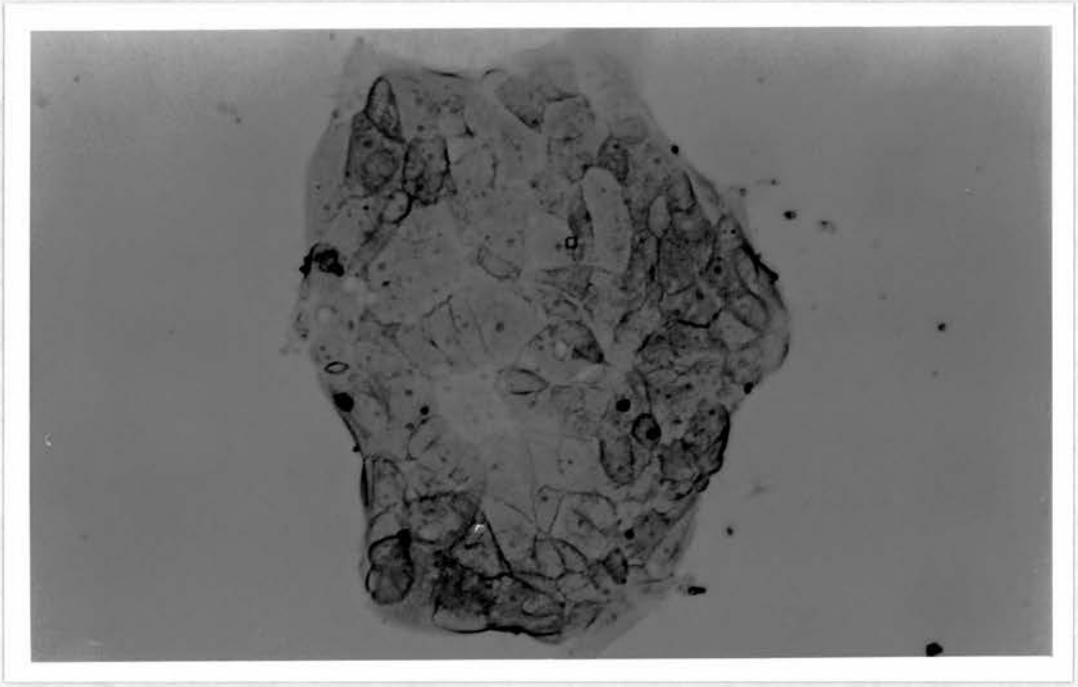


Fig. 119.

TC 21. Anaplastic carcinoma. 15 days culture. Gordon and Sweets' silver impregnation. Note pericellular reticulin deposits. Plain light Original magnification x 150

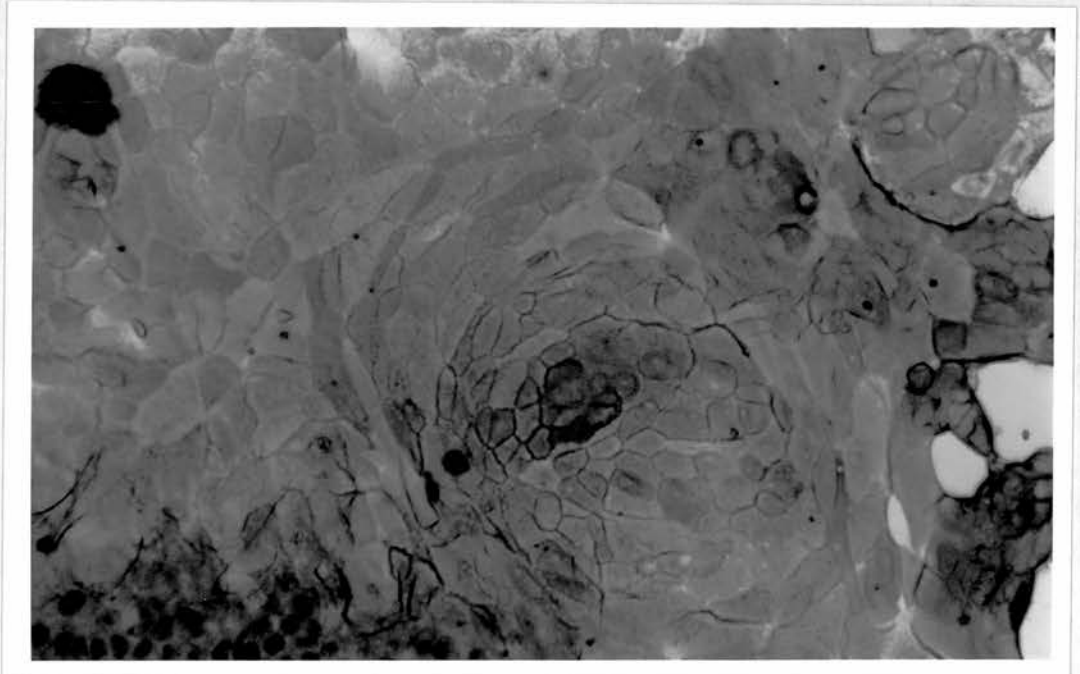


Fig. 120.

TC 42. Anaplastic carcinoma. Variable 'stromal reaction'.

Moderate elastosis. 9 days culture.

Phase contrast. Note pericellular refringence.

Original magnification x 150

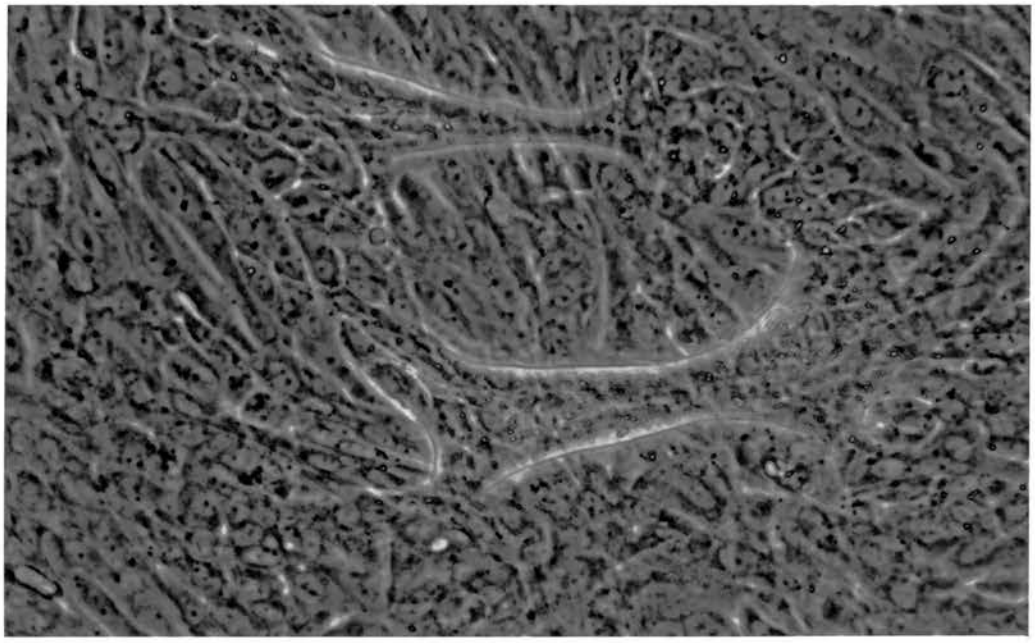


Fig. 121. TC 42.

Silver impregnation. Note coincidence with 'haloes'.

Original magnification x 150

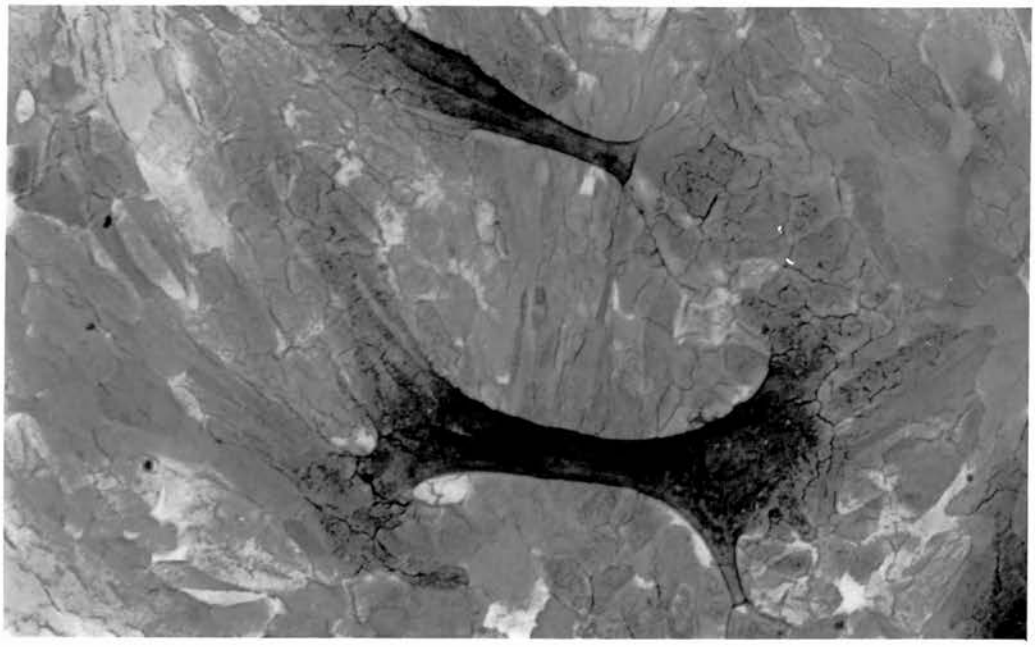


Fig. 122.

TC 42. Anaplastic carcinoma, variable 'stromal reaction', moderate elastosis. 9 days culture.

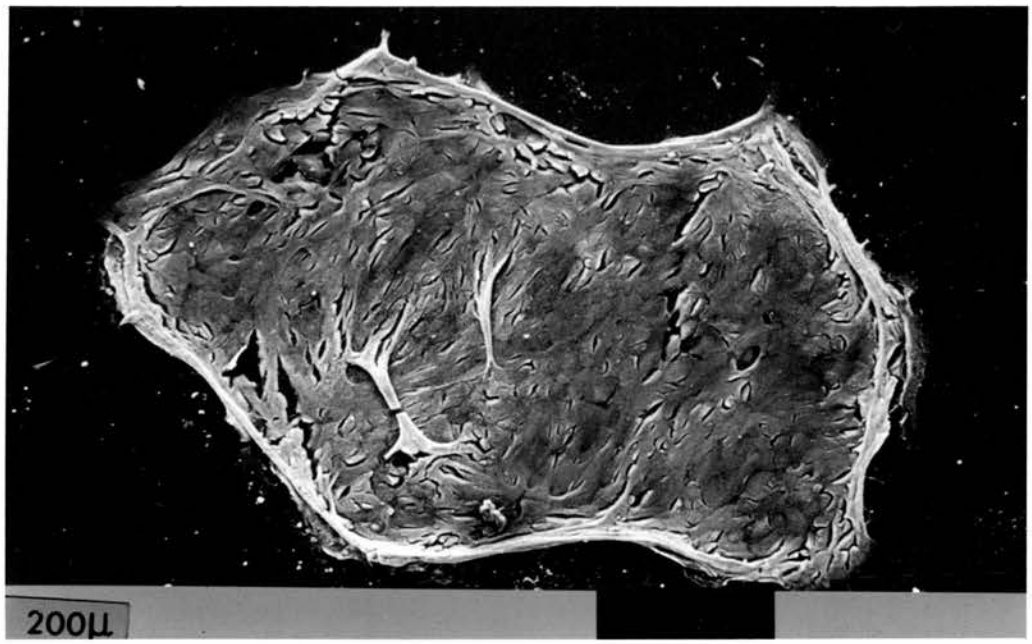
Scanning electron micrograph of the group of cells depicted in Figs. 120 and 121.

Fig. 123.

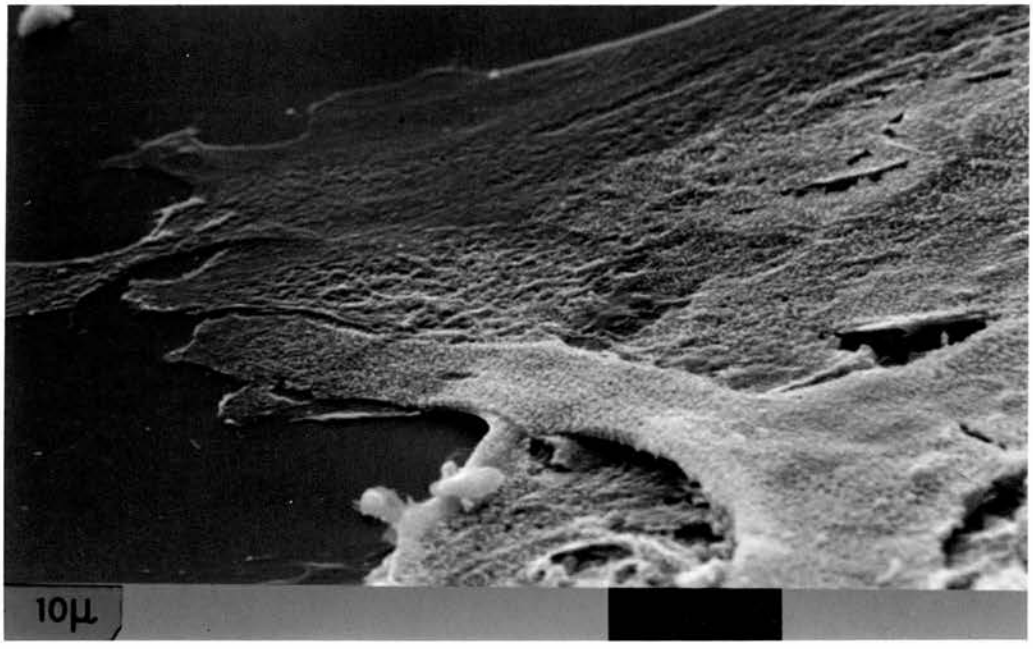
A more detailed view of some of the cells at the edge of the cell pavement.

Following photographic recording of the results, the group of cells from TC 42, depicted in the previous two photomicrographs, was subsequently processed for examination with the scanning electron microscope. Using this method of examination it could be seen that, in those areas where reticulin had been demonstrated histologically and where, viewed with phase contrast microscopy, refractile 'haloes' had been present, there were large quantities of granular material.

Low magnification view of whole cell group. Note lighter areas around edges and across cellular bridges.

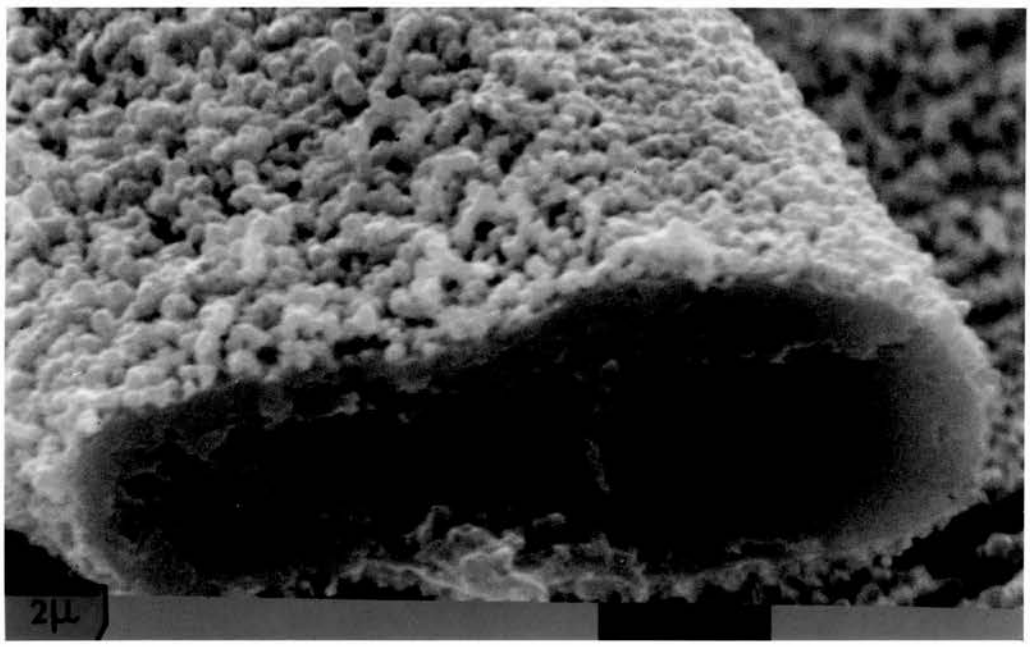


There was a marked variation in the granularity on the surface of the cells.



There was a particularly high concentration of granular material on the cell bridges which crossed the middle of the cell pavement. This was the area which, by phase contrast microscopy, had exhibited the greatest degree of 'shine', and which, on 'staining' for the presence of reticulin, had the highest apparent accumulation. These results suggest that the granules visible with the scanning electron microscope are adherent silver grains deposited during the 'staining' procedure. Confirmation of this supposition could be obtained by X-ray analysis of the cell group to 'map' the distribution of silver.

Fig. 124. High magnification of fractured cell bridge (fractured during processing for examination with the scanning electron microscope) showing high concentration of granular material around the outside.



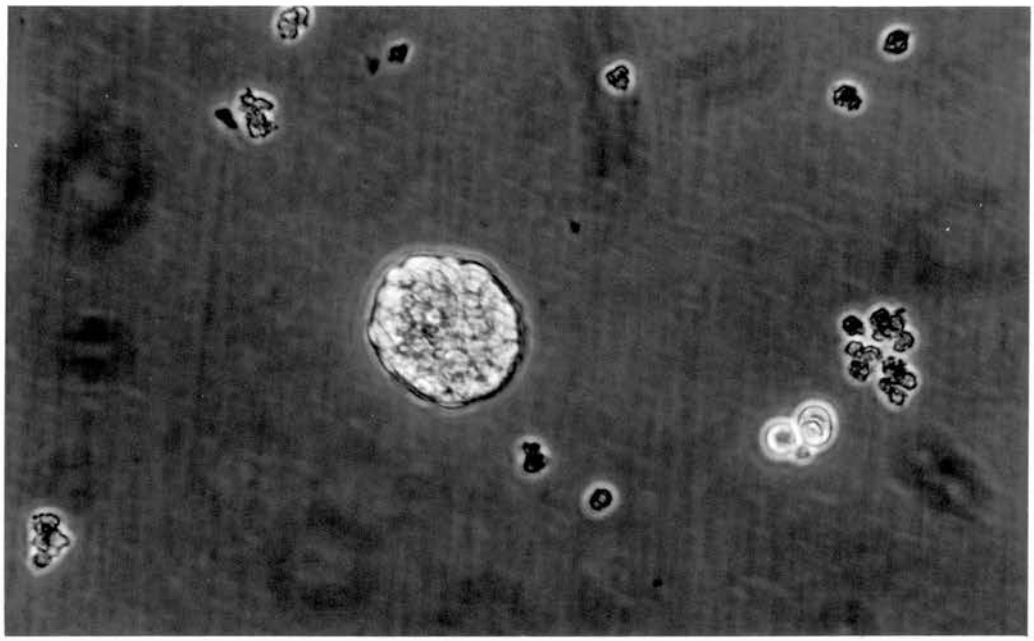
Figs. 125 and 126.

Cellular spheres found in cultures of TC 72, an anaplastic carcinoma with an apocrine component.

Phase contrast. Original magnification x 150

A further area where reticulin was demonstrated was in and around the cell spheres found in cultures of TC 72, an anaplastic carcinoma with an apocrine component. When cultures were prepared from this biopsy the most striking feature of the cultures was the continued presence of balls of apparently viable cells, similar to those obtained from TC 68 (see p. 153), floating in the medium.

TC 72.



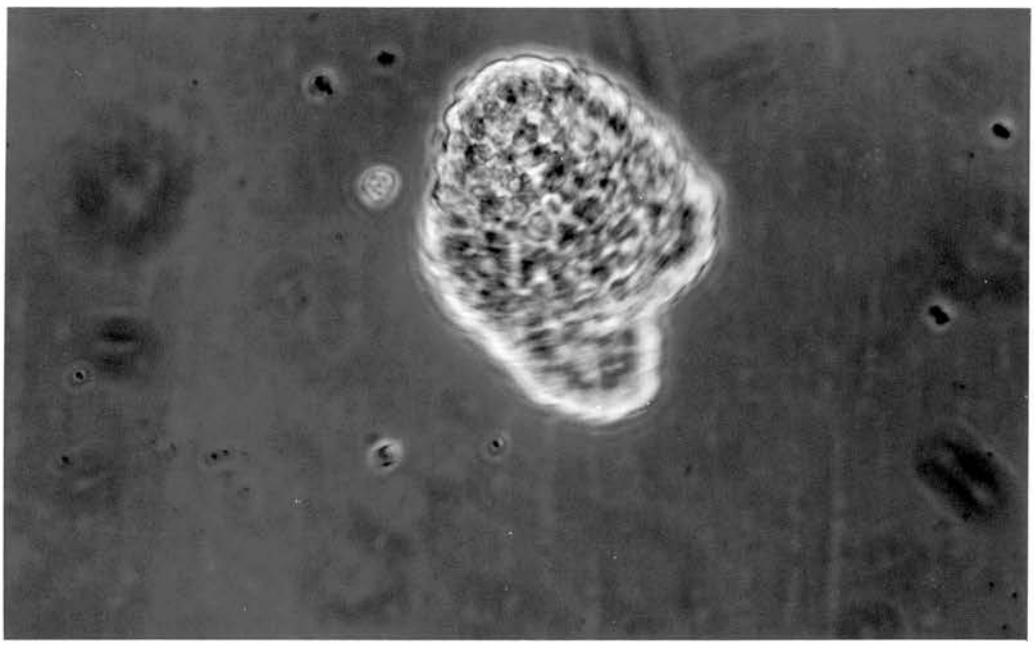


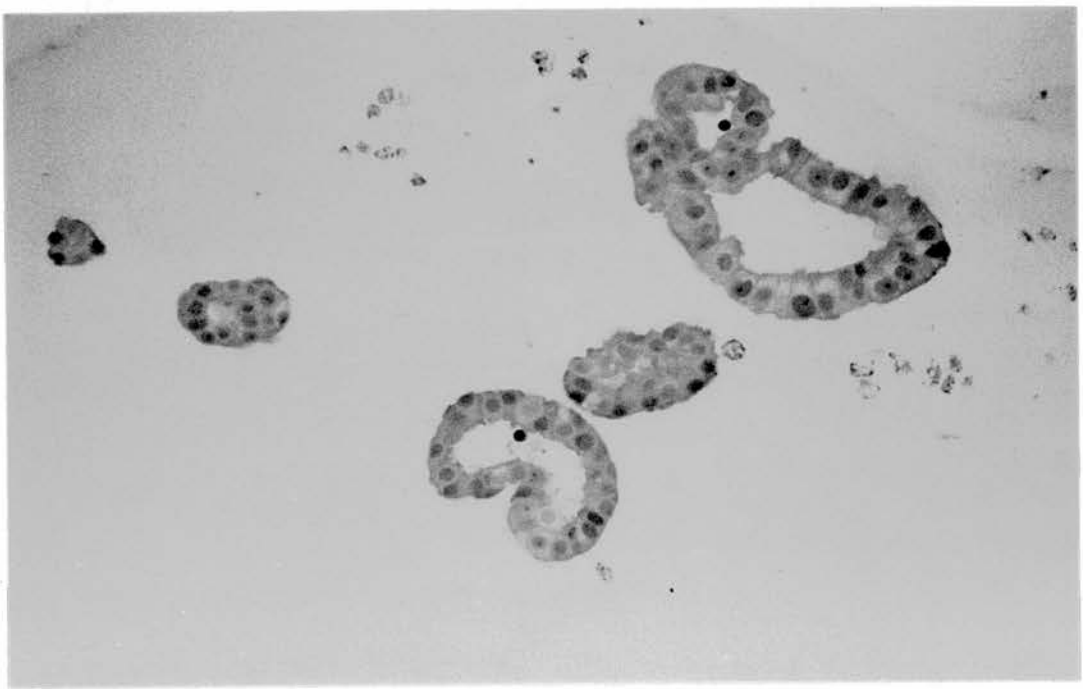
Fig. 127.
Histological sections of spheres as depicted in Figs. 125 and 126 (pp 129).

One month following culture initiation the cell balls were embedded in glycol methacrylate and examined histologically.

The cell spheres were composed of single or double layers of cells around a central lumen. The cells were typically epithelial in appearance with prominent nuclei and a single nucleolus.

2 µm section. Haematoxylin, phloxine & saffron stain.

Original magnification X 72

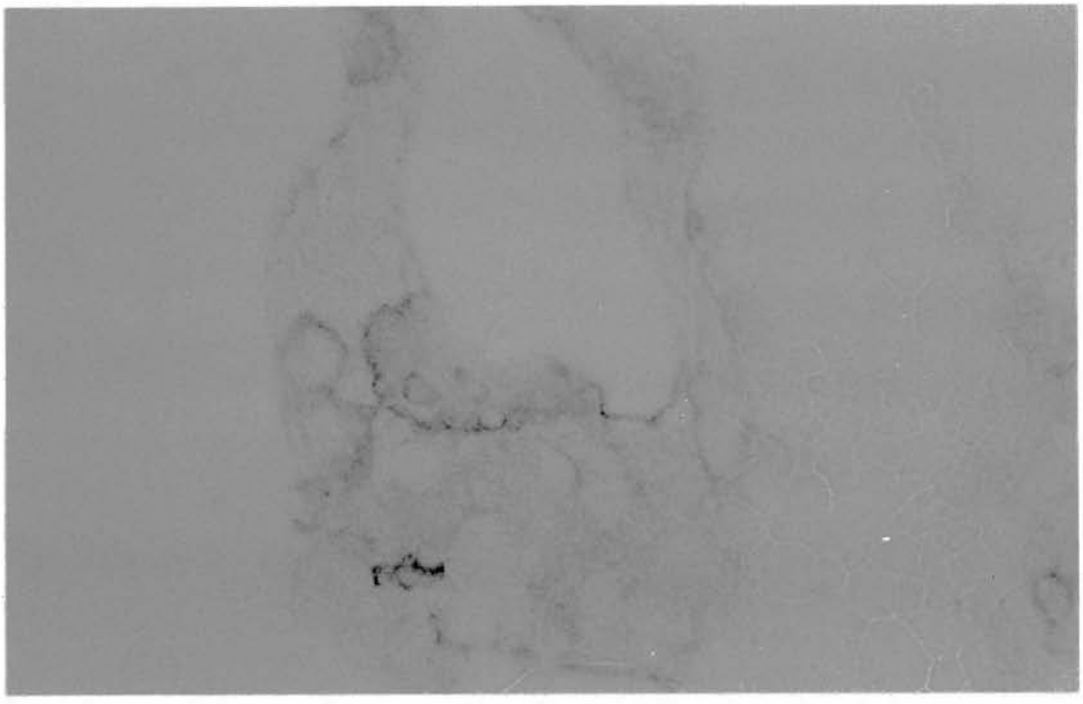


Figs. 128 and 129.

Histological section of cellular spheres found in cultures of TC 72, an anaplastic carcinoma with an apocrine component.

When Gordon and Sweets' silver impregnation technique was applied to sections of the same glycol methacrylate embedded material it could be seen that there were deposits of reticulin in a network around the cells.

Original magnification X 144



Gordon and Sweets' silver impregnation method / tartrazine.

Original magnification X 72

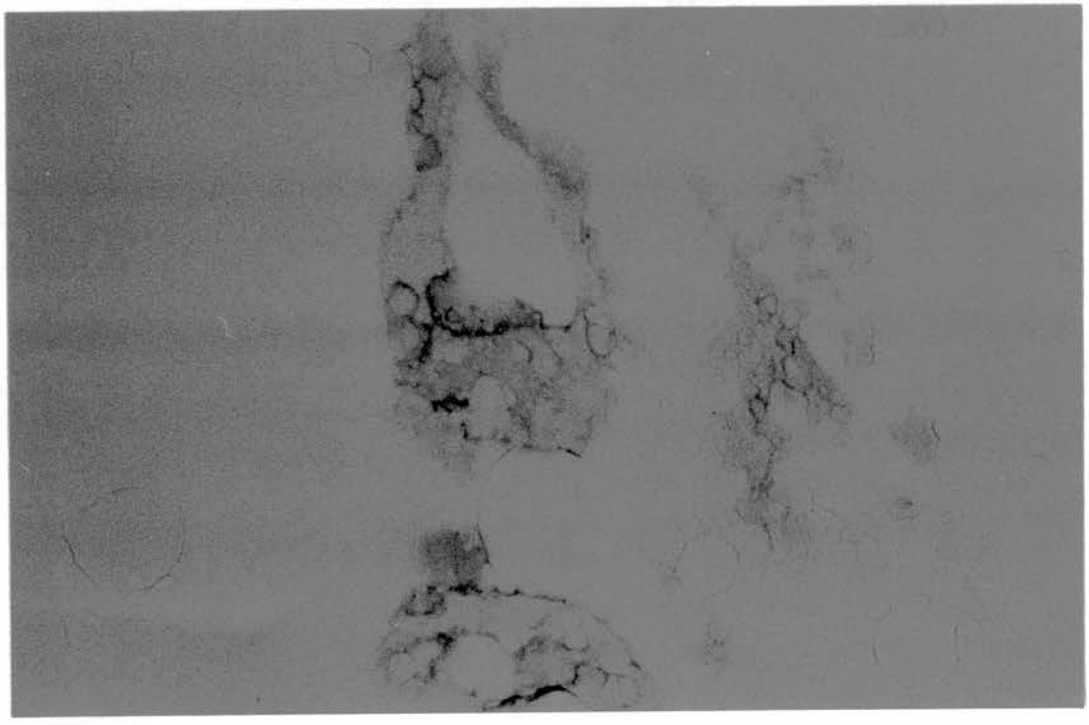
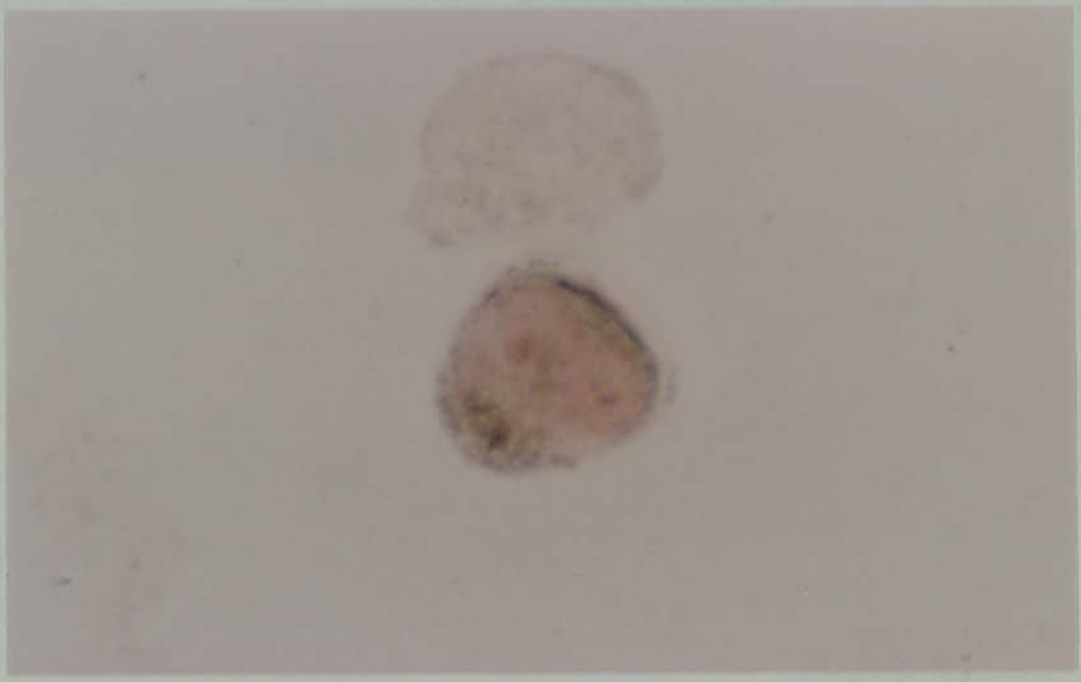


Fig. 130.

Droplet preparation from TC 76, a highly elastotic anaplastic carcinoma with an apocrine component. Note peri-cellular silver deposition indicating the presence of reticulin.

A similar type of reticulin distribution was seen around single cells released from the cell culture into the medium and stained in 'droplet' preparations made on microscope slides.

TC 76. Droplet preparation. Gordon and Sweets' silver impregnation/ neutral red. Original magnification X 360.



Positive identification of peripheral or pericellular reticulin deposits was made in 14 separate cultures of both benign and neoplastic tissue.

The cultures which were tested were:-

TC 2 Fibroadenosis

TC 6 Fibroadenosis

TC 7 Fibroadenosis

TC 19 Fibroadenosis

TC 21 Encephaloid carcinoma

TC 23 Anaplastic carcinoma

TC 33/

TC 33 Anaplastic, small cell carcinoma with moderate elastosis.

TC 41 Anaplastic carcinoma.

TC 42 Anaplastic carcinoma with moderate elastosis.

TC 48 Mucoid carcinoma with co-incident fibrocystic disease.

TC 70 Gynaecomastia.

TC 72 Anaplastic carcinoma with an apocrine component.

TC 75 Anaplastic carcinoma.

TC 76 Highly elastotic anaplastic carcinoma (Droplet preparation).

Cultures were selected for reticulin demonstration on the basis of availability as with many of the cultures there were insufficient flasks to allow fixation and hence termination of the culture.

THE PRODUCTION OF FIBRILLAR RETICULIN

In addition to the type of reticulin deposition described above, in some cultures, notably those in which a more 'fibroblast-like' cell was dominant, fibrils of reticulin positive material were laid down during the culture period.

Those cultures in which this type of reticulin was detected were:-

TC 1 Intraduct carcinoma.

CTH 1 The cell line which arose from TC1.

TC 57 Anaplastic carcinoma.

CTH 7 The cell line which arose from TC 57.

TC 72 Anaplastic carcinoma with an apocrine component.

TC 77 Anaplastic carcinoma.

TC 83 Anaplastic carcinoma, grade II elastosis.

TC 84 Anaplastic carcinoma, large cell type.

Fig. 131.

TC 1. Cells viewed by phase contrast microscopy.

Phase contrast. Original magnification x 150

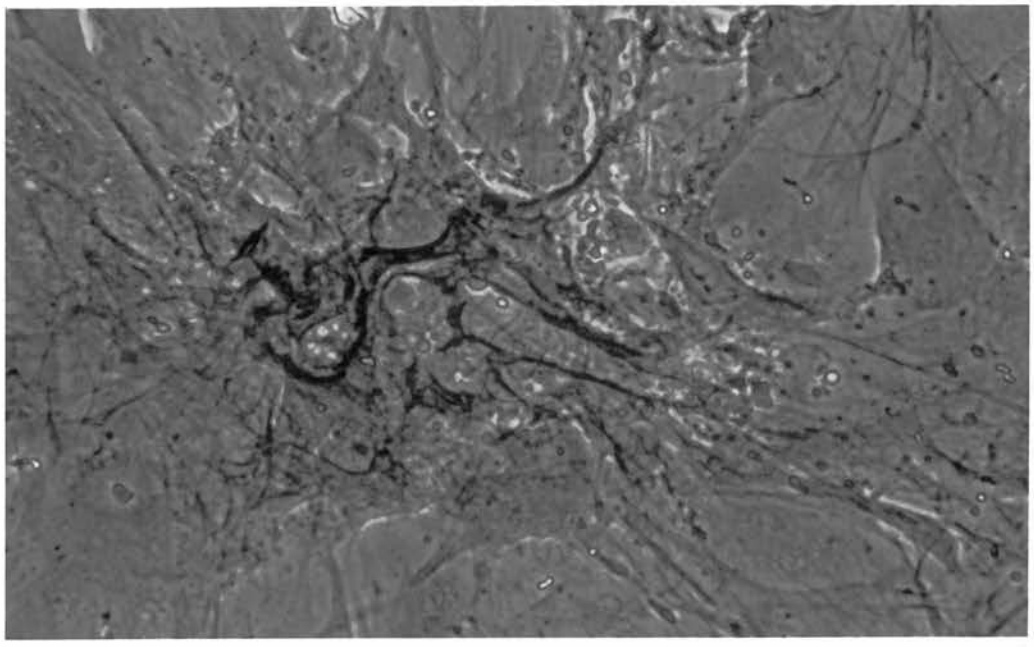


Fig. 132.

The same field as Fig. 131.

Reticulin fibres viewed with plain light.

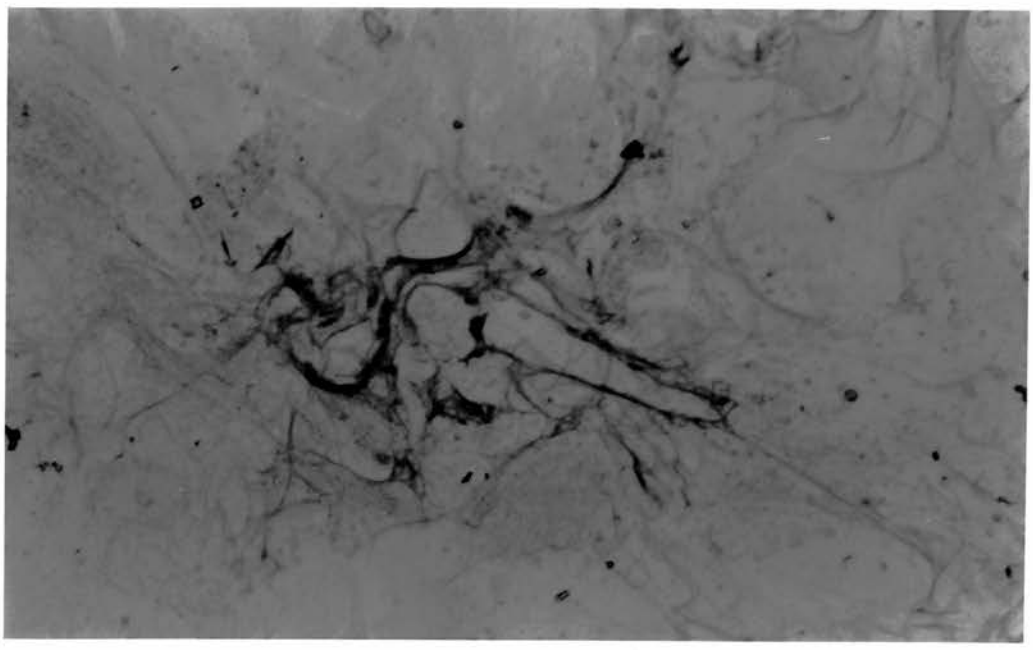


Fig. 134.

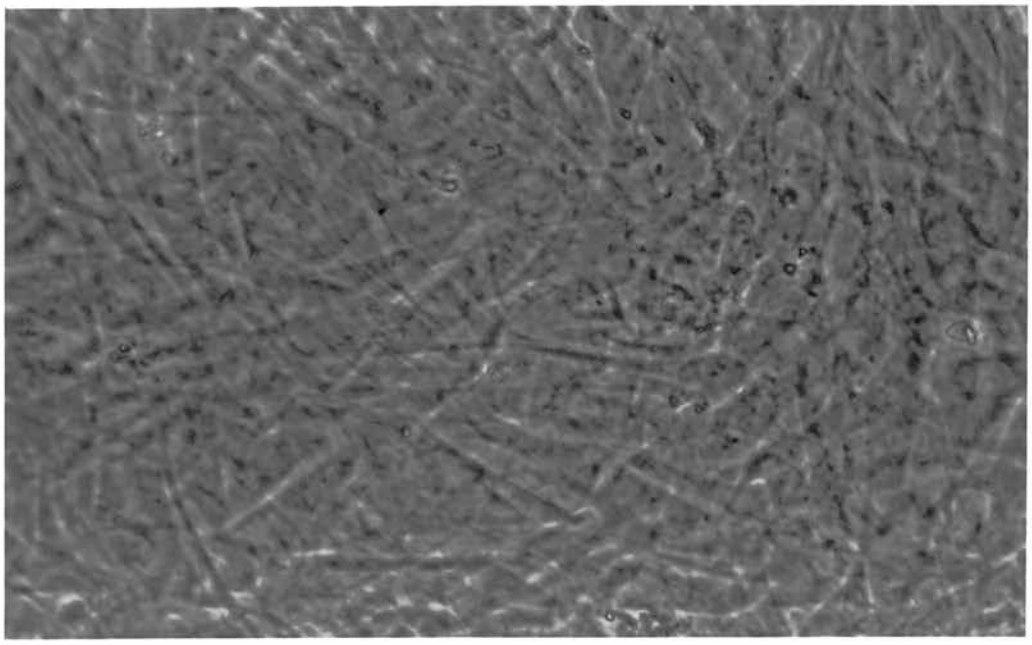
The same field as that shown in Fig. 133. Plain light.

Original magnification x 150

Fig. 133.

CTH 1 cells. The cells of this cell line piled up on one another in many layers.

Phase contrast. Original magnification x 150



Fine reticulin fibres laid down on the surface of the CTH 1 cells. This cell line retained the facility of reticulin production through 9 passages over a period of 5 months when incubator failure destroyed the cells.

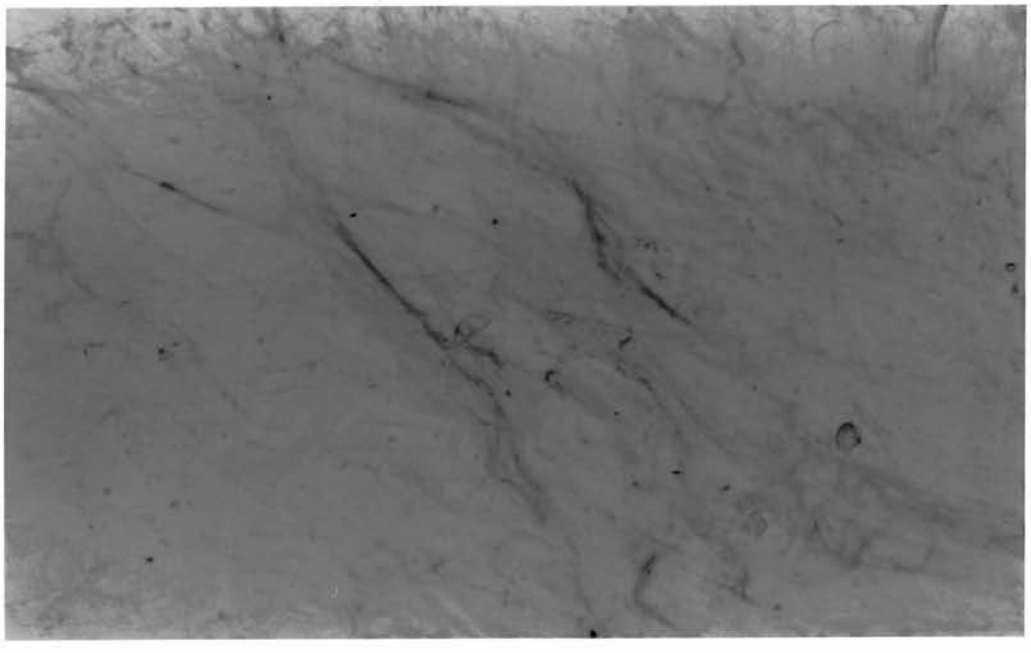


Fig. 136.

Reticulin fibre network as demonstrated by the silver impregnation technique.

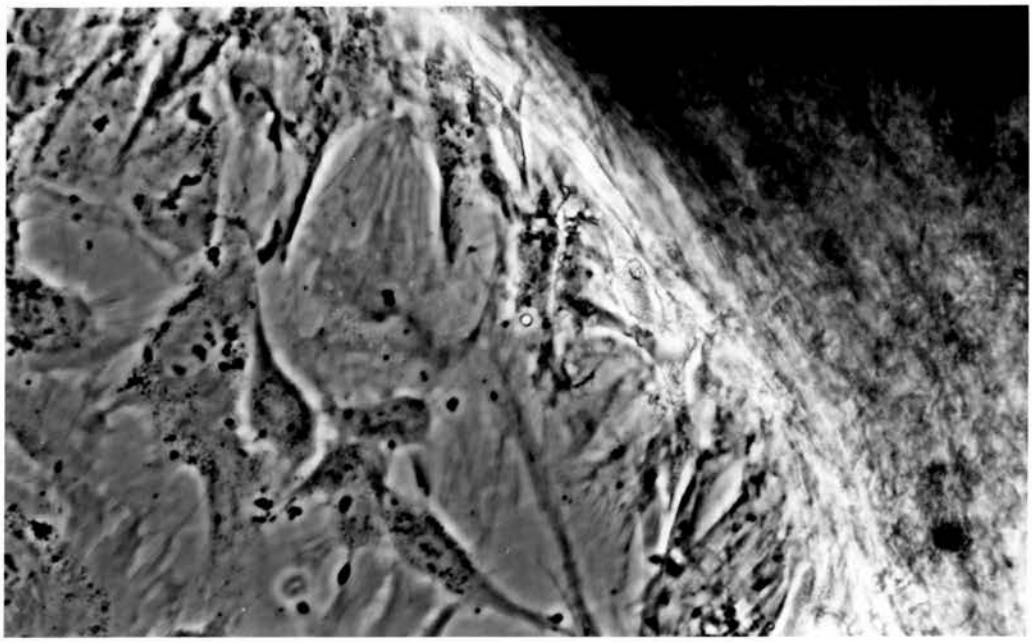
Plain light. Original magnification x 150

Fig. 135.

TC 83. Anaplastic carcinoma with grade II elastosis.

The fibroblast-like cells in these cultures piled up into ridges and it was in the area of these ridges that networks of reticulin fibres were demonstrated using the Gordon and Sweets' silver impregnation technique.

Phase contrast. Original magnification x 150



Reticulin fibre network.

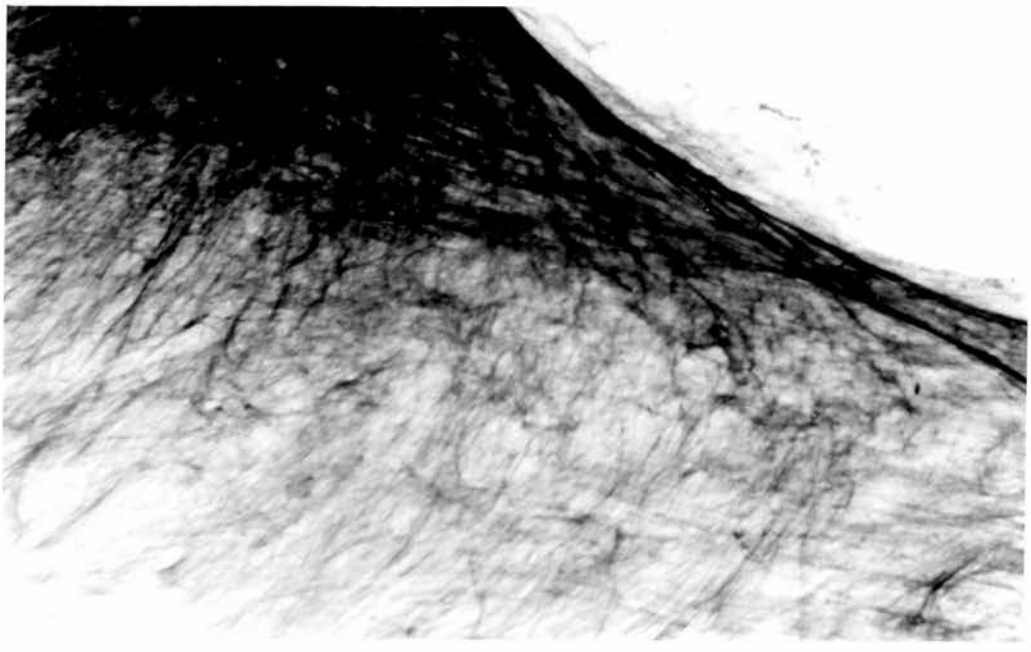


Fig. 137.

Histological section of cell aggregation.

THE PRODUCTION OF CONNECTIVE TISSUE BY PUTATIVE MYOFIBROBLASTS

A further area where reticulin production was demonstrated was within the contracted 'lumps' of tissue which formed during the growth of cells thought to be myofibroblasts.

The myofibroblast was described in Section I and was shown to be associated with the deposition of elastin in culture.

'Fibroblast-like' cells from several other, non-elastotic, tumour cultures contracted into ridges and 'lumps' and representatives of these were taken and processed histologically. Cells of this type were shown to be associated with reticulin, collagen and elastin.

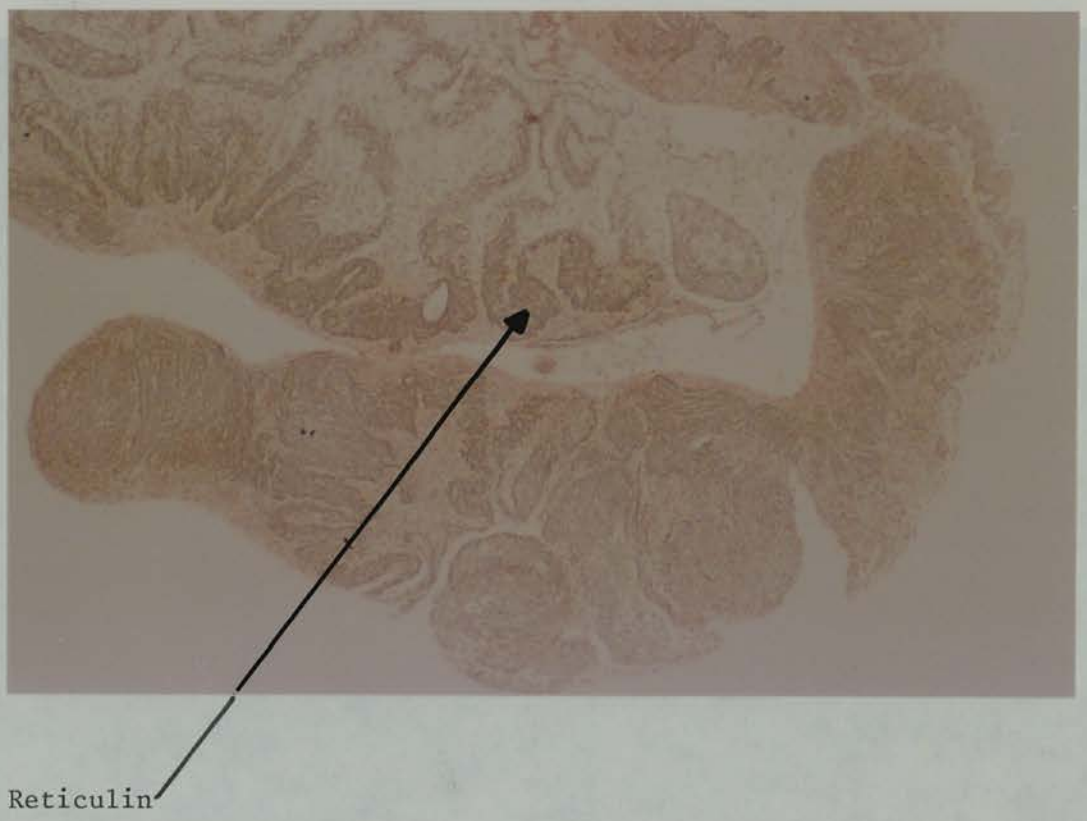
TC 85. Anaplastic carcinoma of large cell type. Gordon and Sweets' silver impregnation for the demonstration of reticulin / neutral red. Original magnification. X 36
50 days culture.

Fig. 138.

Histological section of cell aggregation.

CTH 7 (derived from TC 57 - Anaplastic carcinoma) Pass 3, 13 days post pass. Gordon and Sweets' silver impregnation for the demonstration of reticulin / neutral red.

Original magnification X 14.4



Collagen was also demonstrated in lumps of this type using the trichrome stain: Haematoxylin, phloxine and saffron.

Fig. 139.

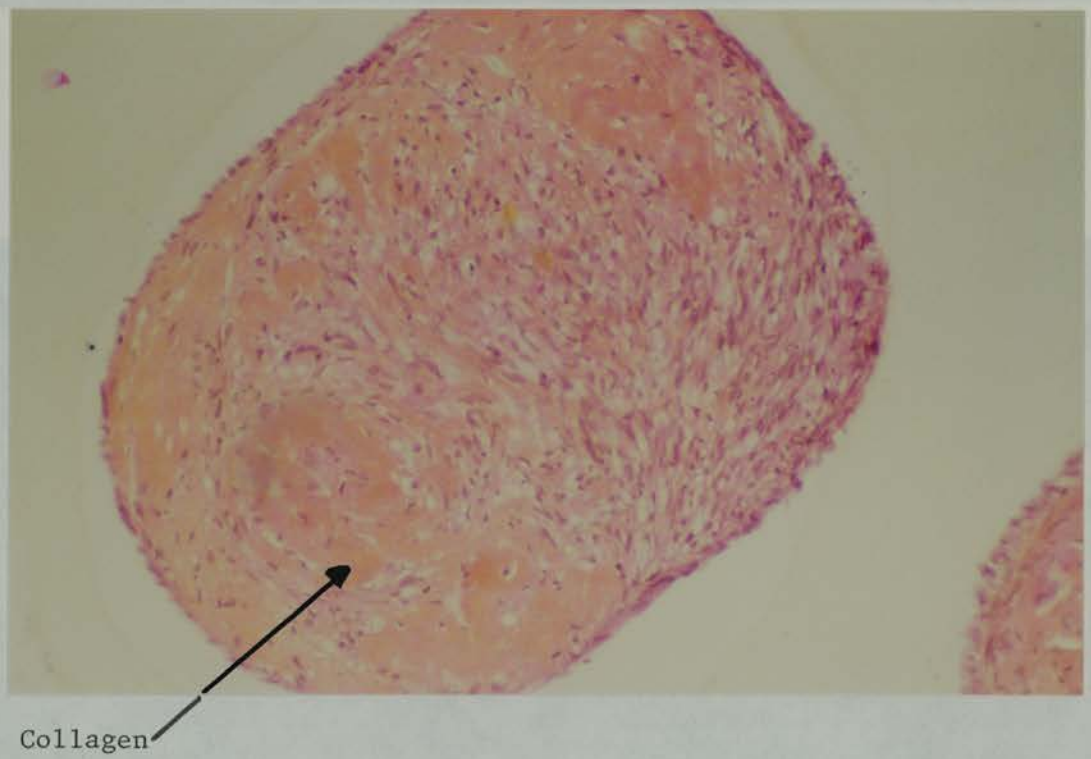
Histological section of cellular aggregation.

Fig. 140.

Detailed view of Fig. 139.

TC 57. Anaplastic carcinoma. Trichrome stain.

58 days culture. Original magnification X 36



TC 57. Original magnification X 144

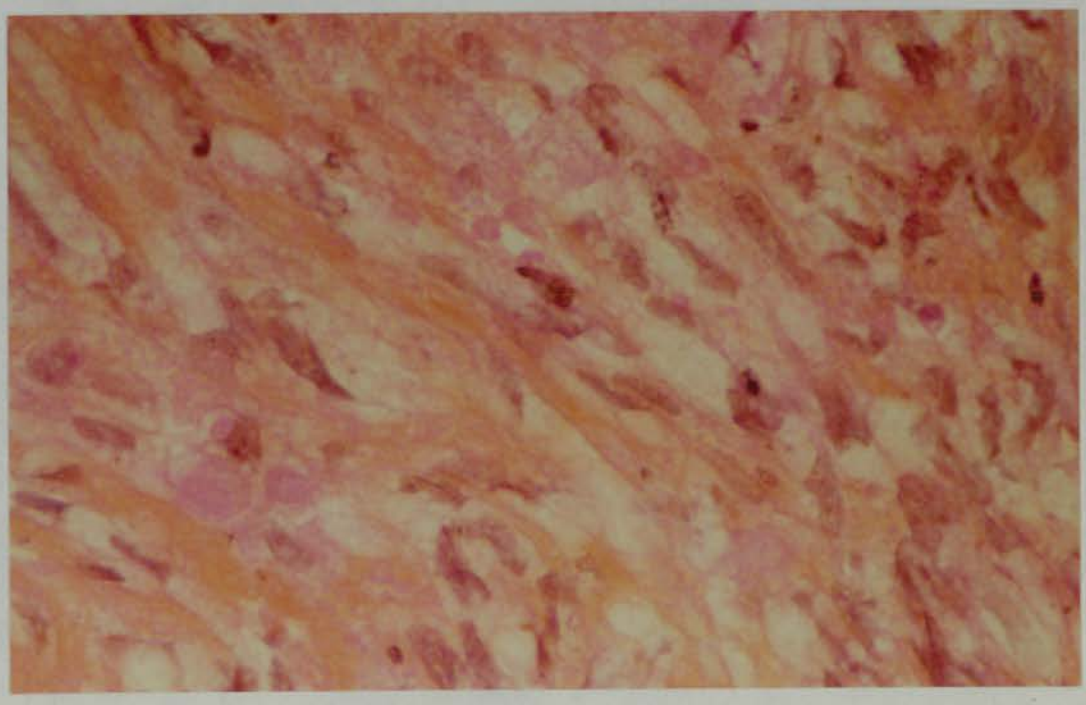


Fig. 141.

Detailed view of Fig. 139.

TC 57. Original magnification X 144

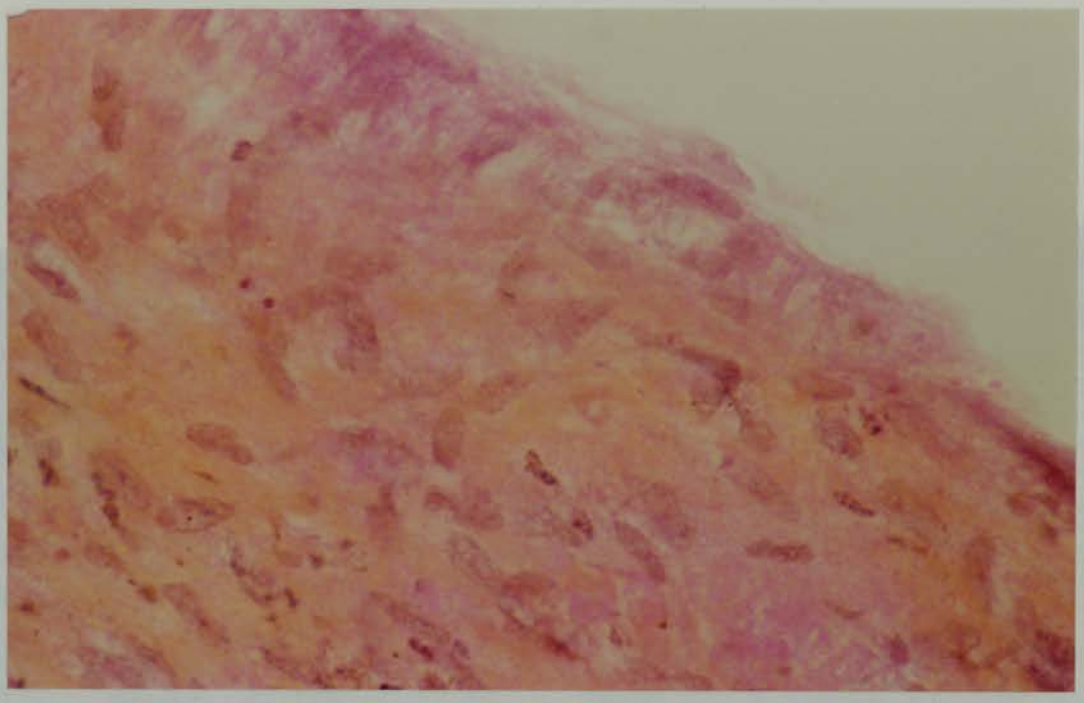


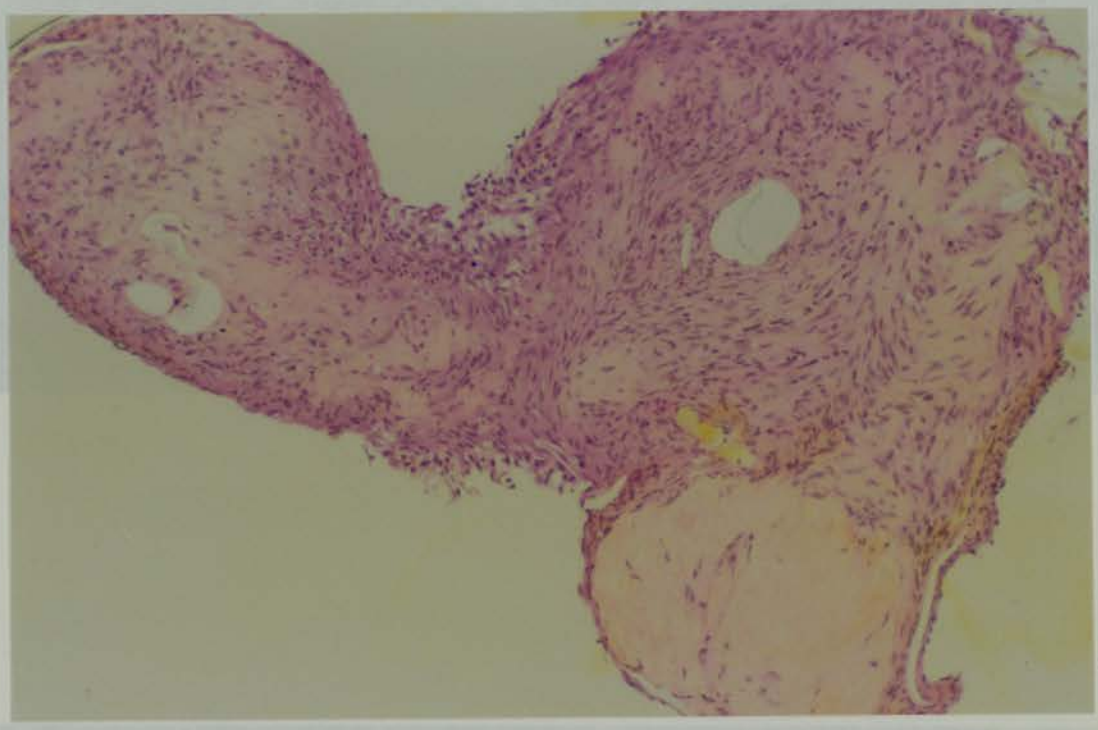
Fig. 142.

Histological section of cellular aggregation.

Fig. 143.

Histological section of cellular aggregation.

TC 78 Lobular carcinoma with a small amount of elastosis. Trichrome stain. 76 days culture. Original magnification X 36



TC 85. Anaplastic carcinoma of large cell type. Trichrome stain. 50 days culture. Original magnification X 14.4

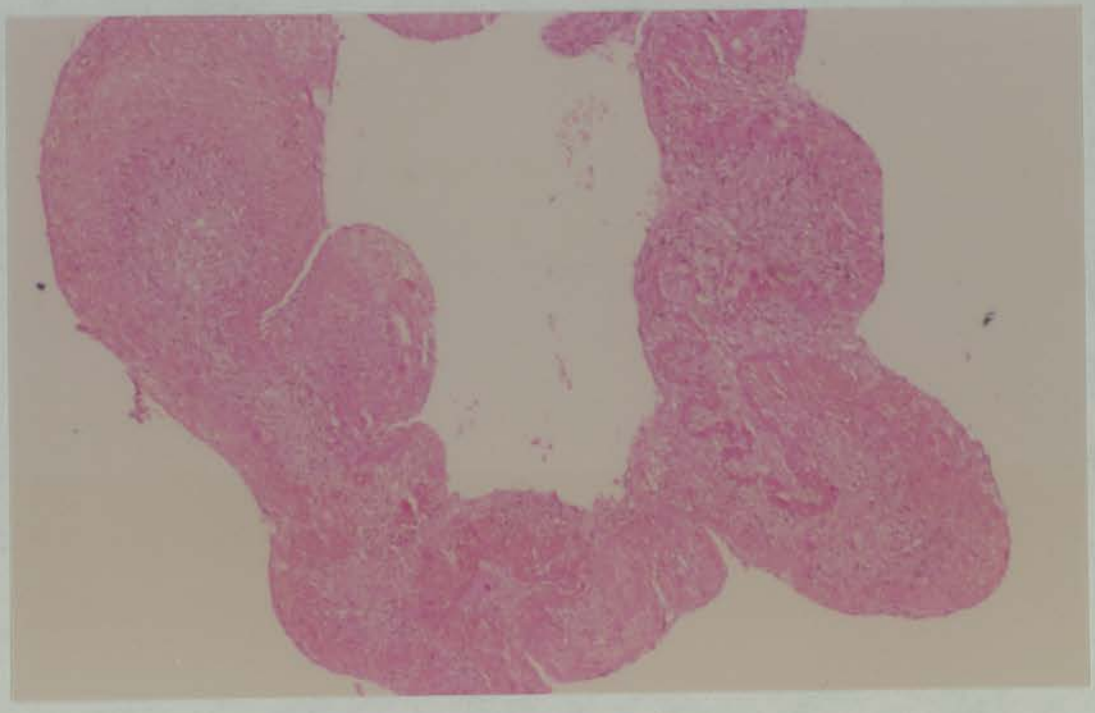
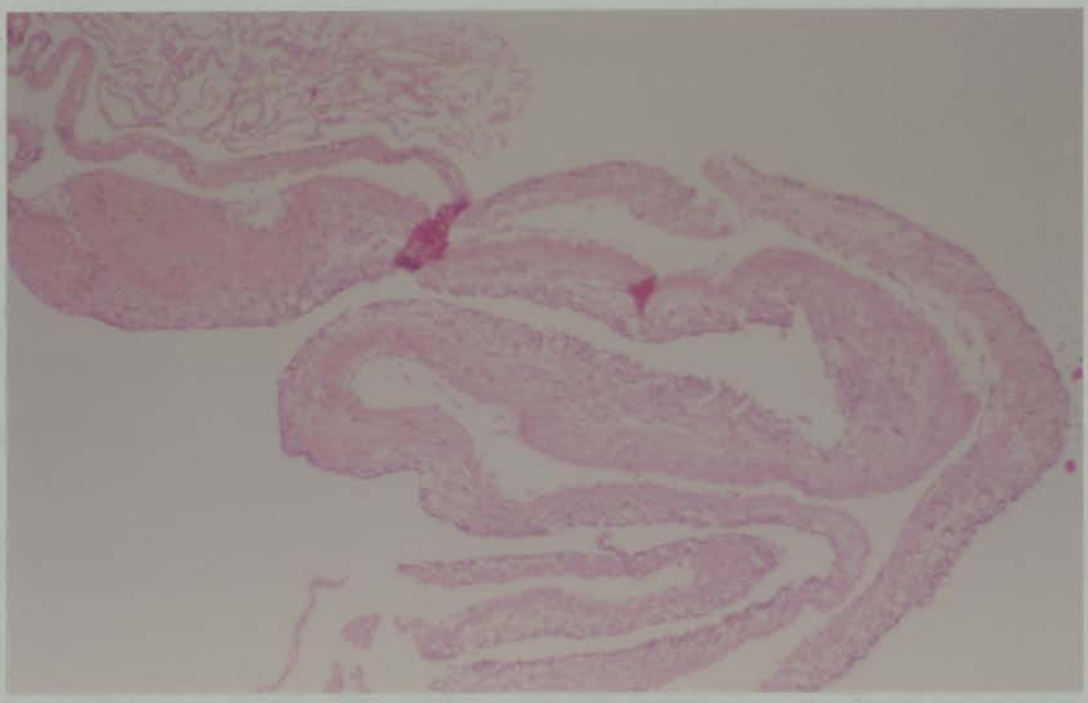


Fig. 144.

Histological section of cellular aggregation.

There was also a demonstrable quantity of collagen, together with the elastin already described (pp 101) in the cell aggregates which formed in cultures from TC 80, a highly elastotic anaplastic carcinoma. This is, perhaps, only to be expected as the admixture of collagen with elastin is a feature of the highly elastotic tumour architecture.[153]

TC 80. Highly elastotic anaplastic carcinoma. Haematoxylin, phloxine and saffron. Original magnification X 14.4



SECTION III

CULTURAL CHARACTERISTICS OF THE APOCRINE VARIANT OF MAMMARY CARCINOMA

One type of tumour which could be separated both morphologically and behaviourally from the majority of those cultured was the apocrine variant of mammary carcinoma.

The original diagnosis which accompanied the tissue specimens contained no information regarding the presence of cells of apocrine type, possibly because of the supposed lack of clinical significance assigned to these cells[107]. The identity of the cells in cultures of mammary tissue emerged only after review of the original biopsy showing the presence in the tumour of cells of apocrine type.

Initially the identification of apocrine cells was made in one culture because of their characteristic behaviour and appearance.[152] Subsequently two further growth characteristics separated this type of tumour from the remainder cultured.

The diagnostic cultural features identified were:-

i) Characteristic cell morphology.

ii) The occurrence of cellular "spheres".

iii) The occurrence of "globules" in areas of concentrated cell growth.

In addition, a rare feature noted only in cultures of tumours of apocrine type was the occurrence of a peripheral palisade of cells at the edge of a cell group.

i) Cellular Morphology and Behaviour.

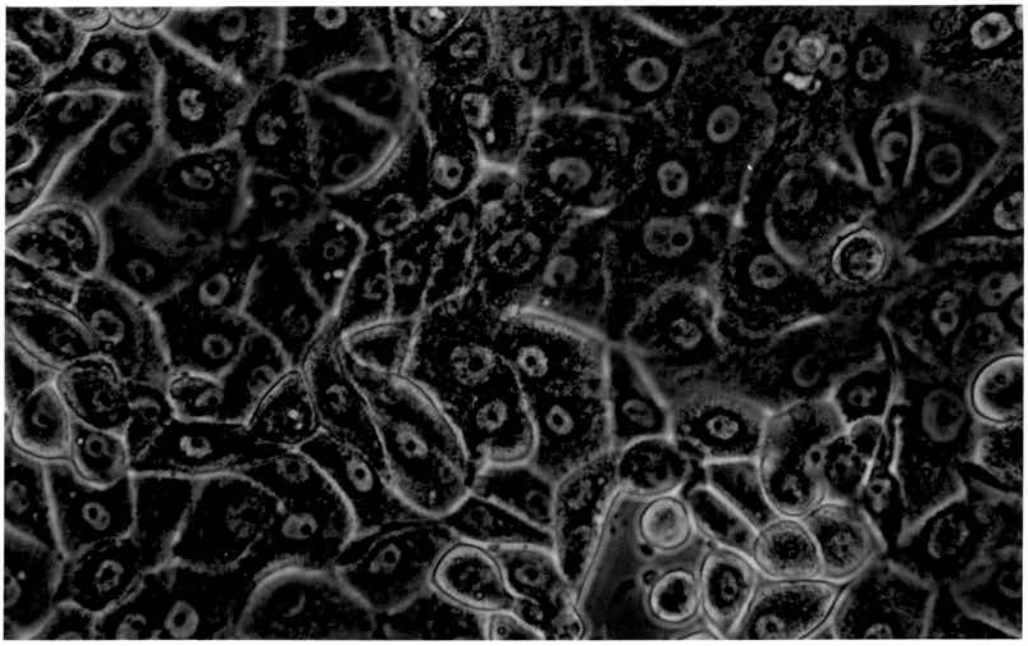
The initial behaviour of apocrine cells was similar to that of other epithelial tissue and monolayers of typically polygonal cells were established on the floor of the flask. The cells were large with abundant, coarsely granular perinuclear cytoplasm, pale nuclei and prominent nucleoli.

Fig. 145.

TC 51. Apocrine variant of mammary carcinoma. 9 days culture.

Phase contrast. Original magnification x 150

TC 51. Typical field. 9 days culture.



Very soon after the establishment of the cell colonies a striking phenomenon was observed in that large quantities of cells and "debris" were shed into the medium. Release of the material appeared to lead to vacuoles in the cell pavement.

Fig. 146.
TC 51. A typical clump of floating cells (see p.152) together with the single cells and "debris" shed into the medium.
Phase contrast. Original magnification x 150

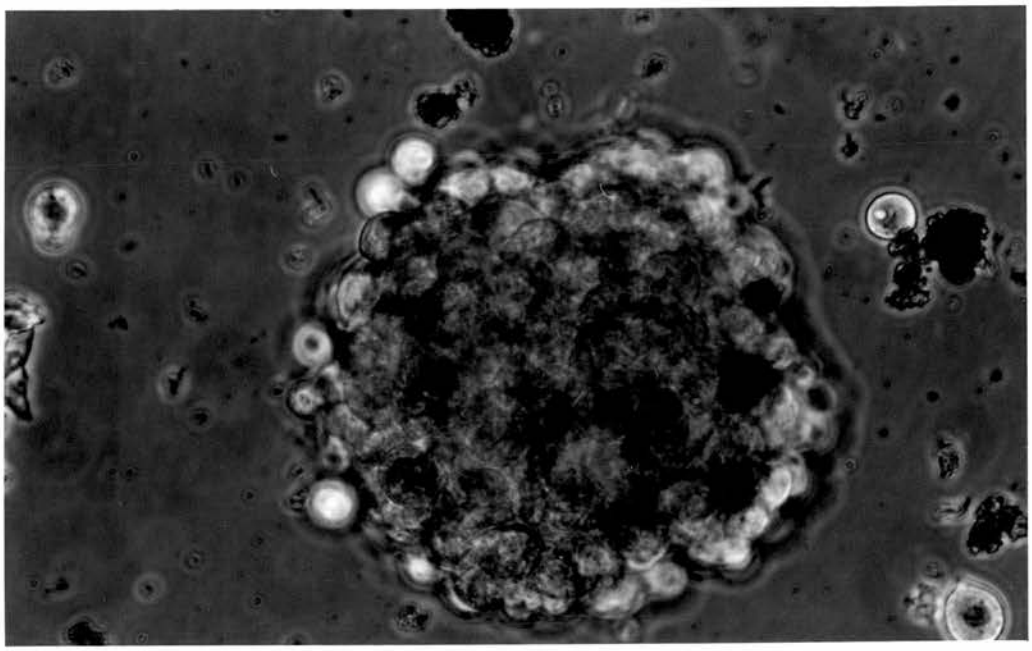
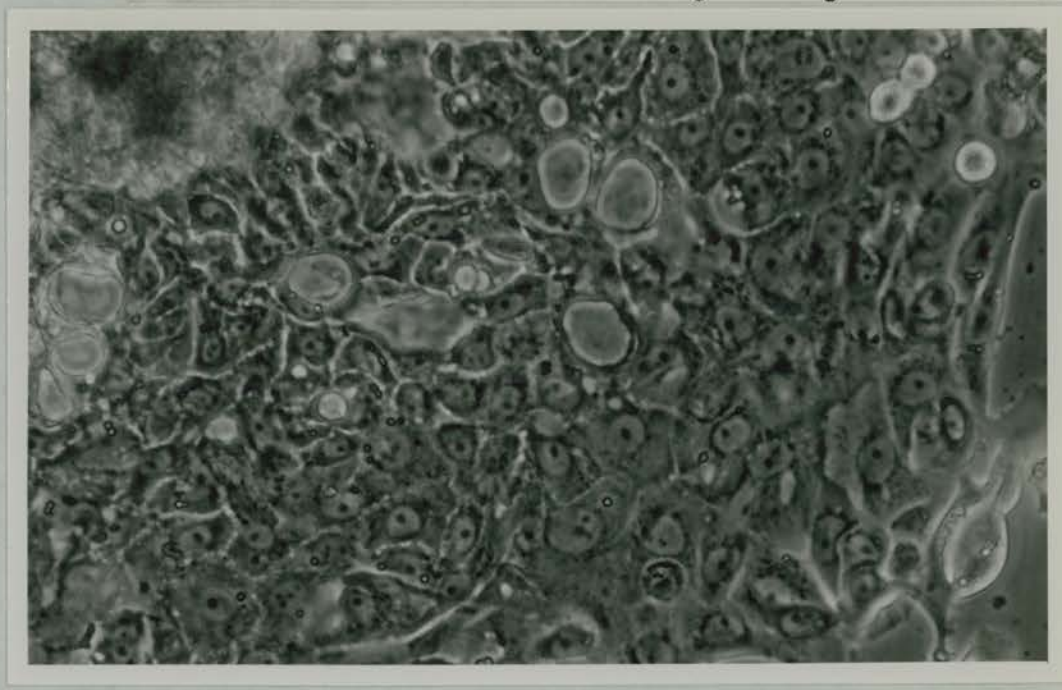


Fig. 147.

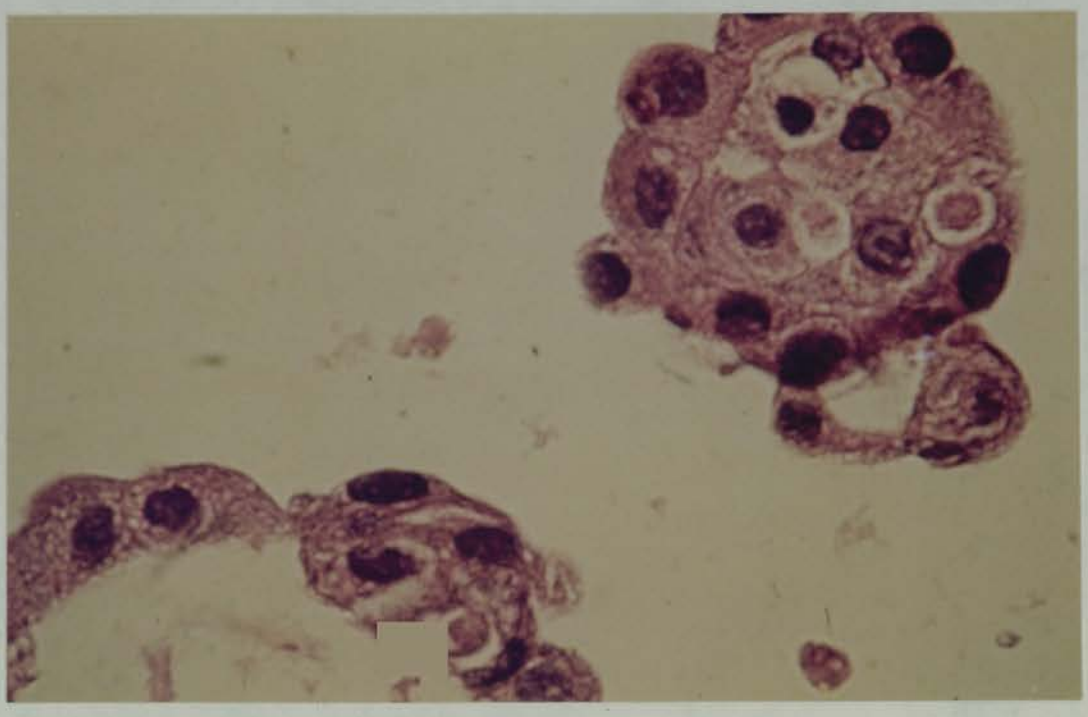
TC 51. 5 days culture, showing vacuolated areas of the cell monolayer.

Phase contrast. Original magnification x 150



In order to determine the nature of the exfoliated material samples of medium were centrifuged and agar blocks were prepared from the sedimented material (pp 34). Examination of the resultant sections revealed cytoplasmic fragments, individual cells and clumps of cells of characteristic apocrine morphology.

Fig. 148. TC 51. Exfoliated cell clumps in paraffin section illustrating clearly the apocrine morphology. Original magnification X 144



Review of the biopsy revealed a high proportion of cells of apocrine type and suggested that it was, indeed, this type of cell which was growing in culture.

Fig. 149. TC 51. A typical field from the diagnostic biopsy showing clearly the apocrine characteristics. H & E Original magnification X 72

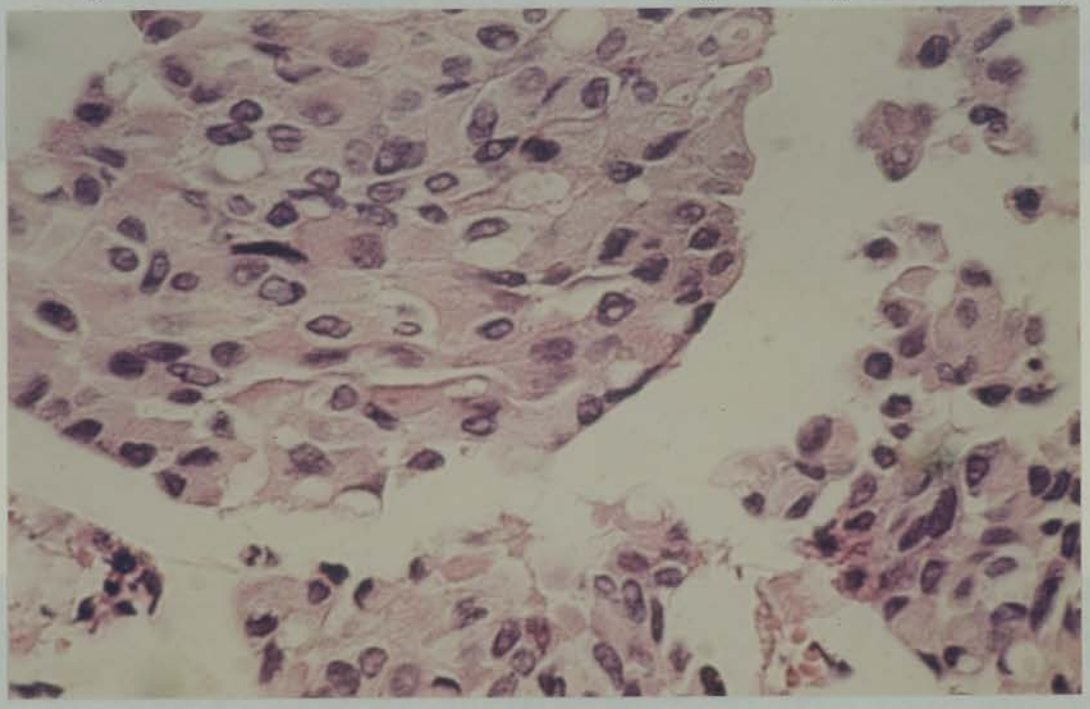


Fig. 150 TC 51. Another field from the biopsy. H & E Original magnification X 72

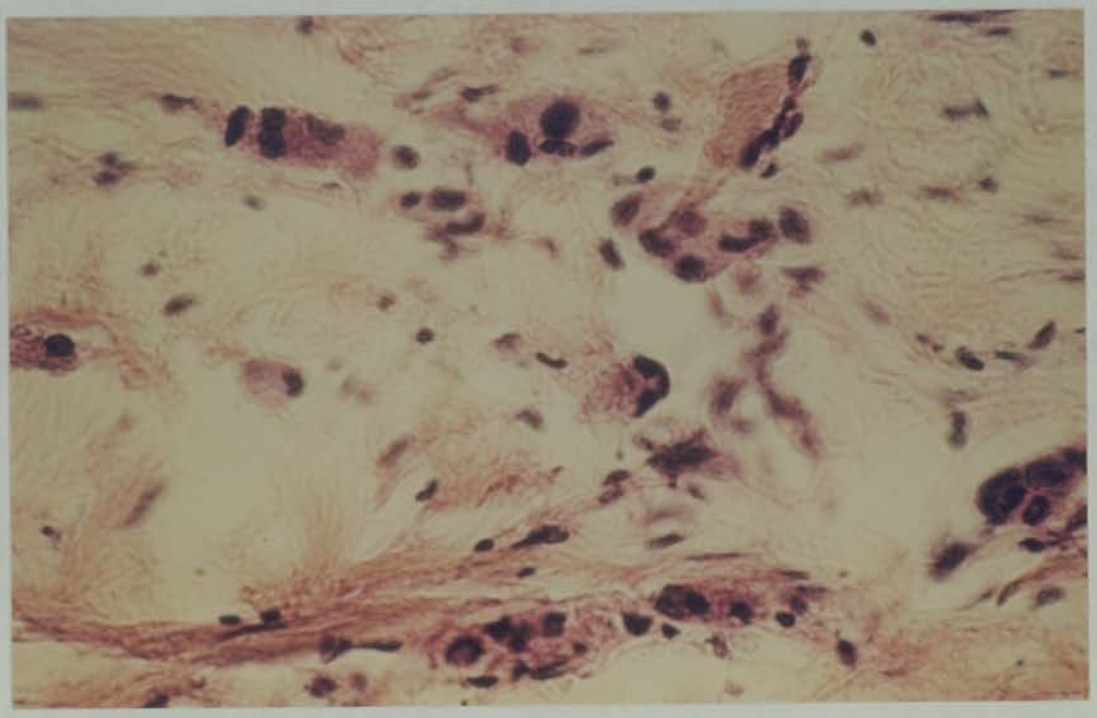


Fig. 151.

TC 76. Highly elastotic anaplastic carcinoma. 20 days culture. Low power view of granular apocrine cells originally reported in the context of elastotic cells. See Figs. 8, 9 and 10 (pp 52 and 53). Phase contrast. Original magnification x 150

Following the demonstration of functional apocrine cells in culture, all the cultures were reviewed for the presence of cells of similar type. Positive identification, together with subsequent confirmation by review of the biopsy, was made in 20 cultures.

Cells from a few representative cultures are illustrated:-

TC 76. Highly elastotic anaplastic carcinoma. 20 days culture.

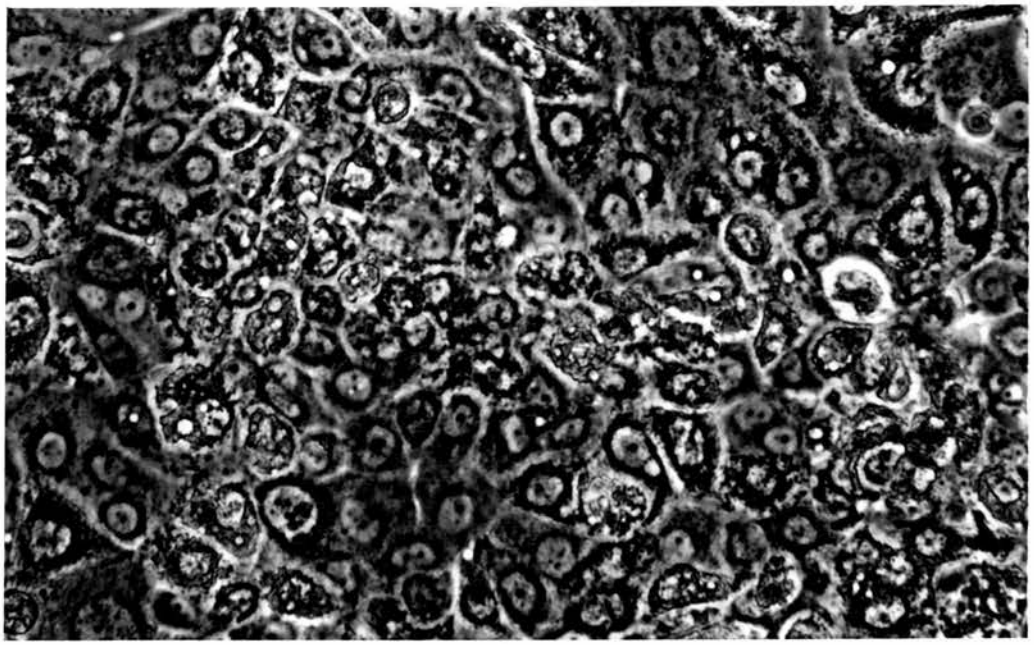


Fig. 152.
TC 76. 12 days culture. Original magnification X 300.
Phase contrast.

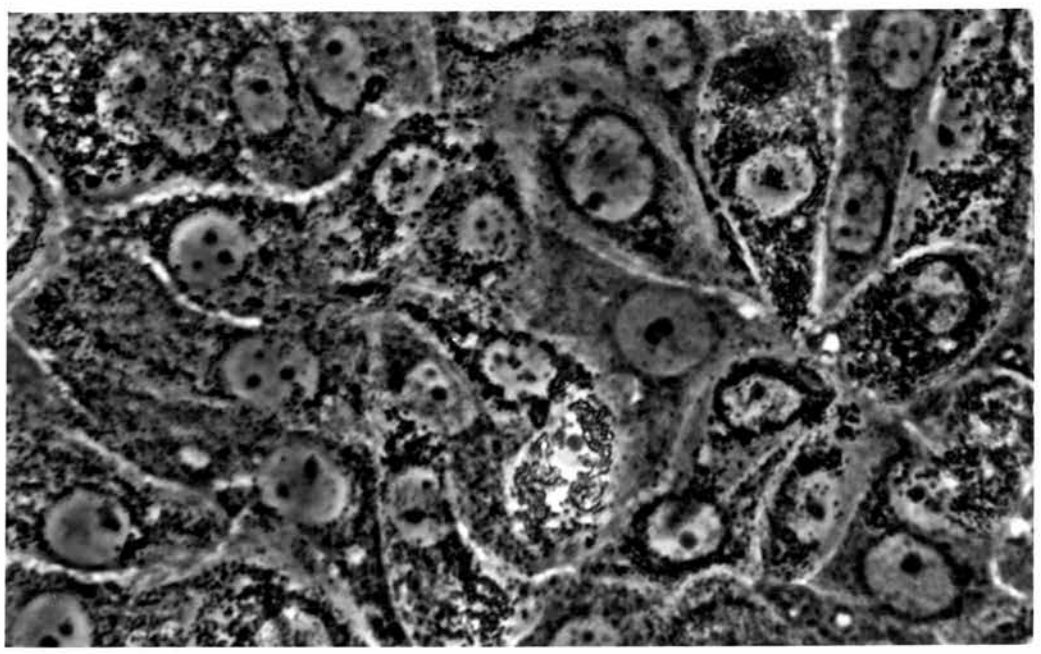


Fig. 153.

TC 76. Highly elastotic anaplastic carcinoma. 22 days culture, showing large and small vacuoles. Original magnification X 300.

Phase contrast.

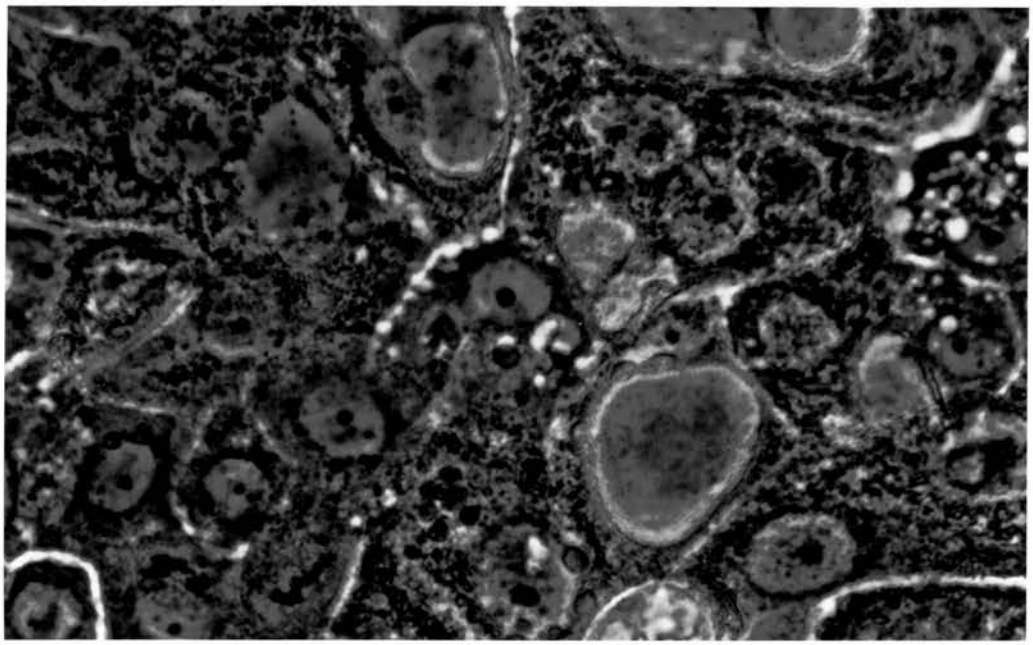


Fig. 154.

TC 26. Anaplastic mucoid carcinoma which, on review of the biopsy, was found to contain a high proportion of cells of apocrine type. 2 days culture.

Phase contrast. Original magnification x 150

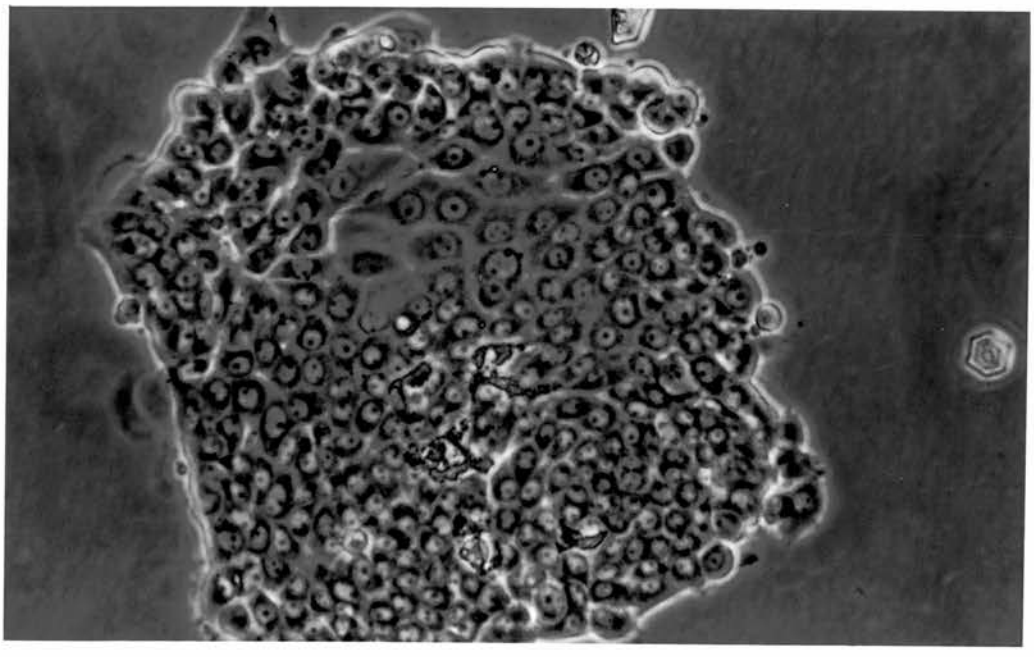


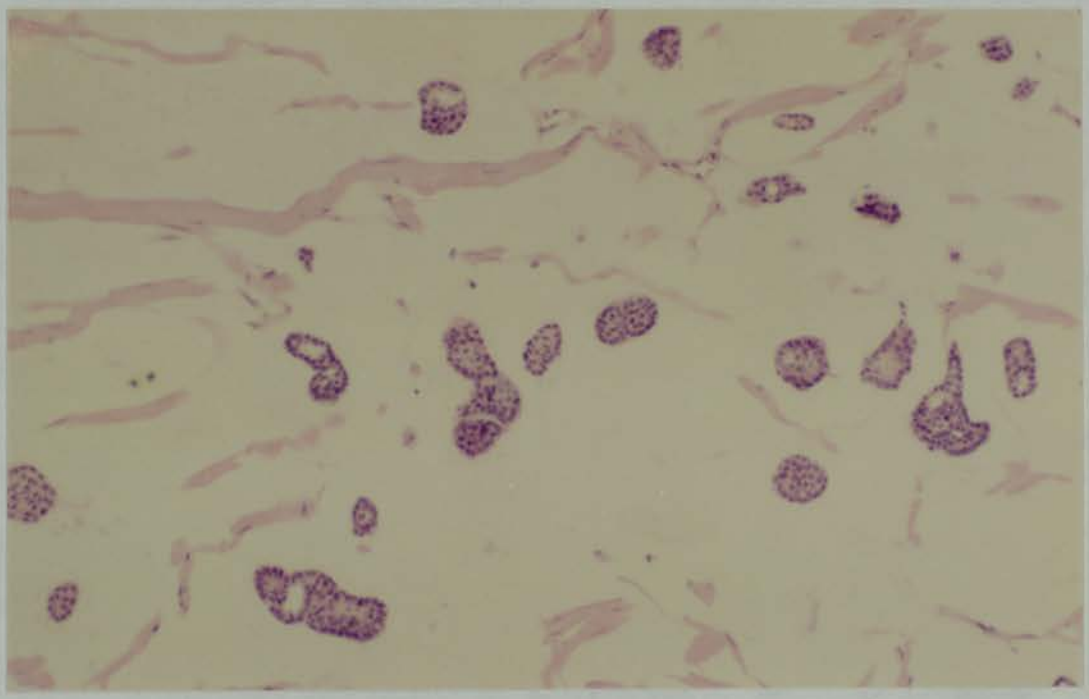
Fig. 156.

Typical groups of apocrine cells.

Fig. 155.

TC 26. Anaplastic mucoid carcinoma. Typical fields from the biopsy showing a high proportion of cells of apocrine morphology.

H & E Original magnification X 36



TC 26. H & E Original magnification X 144

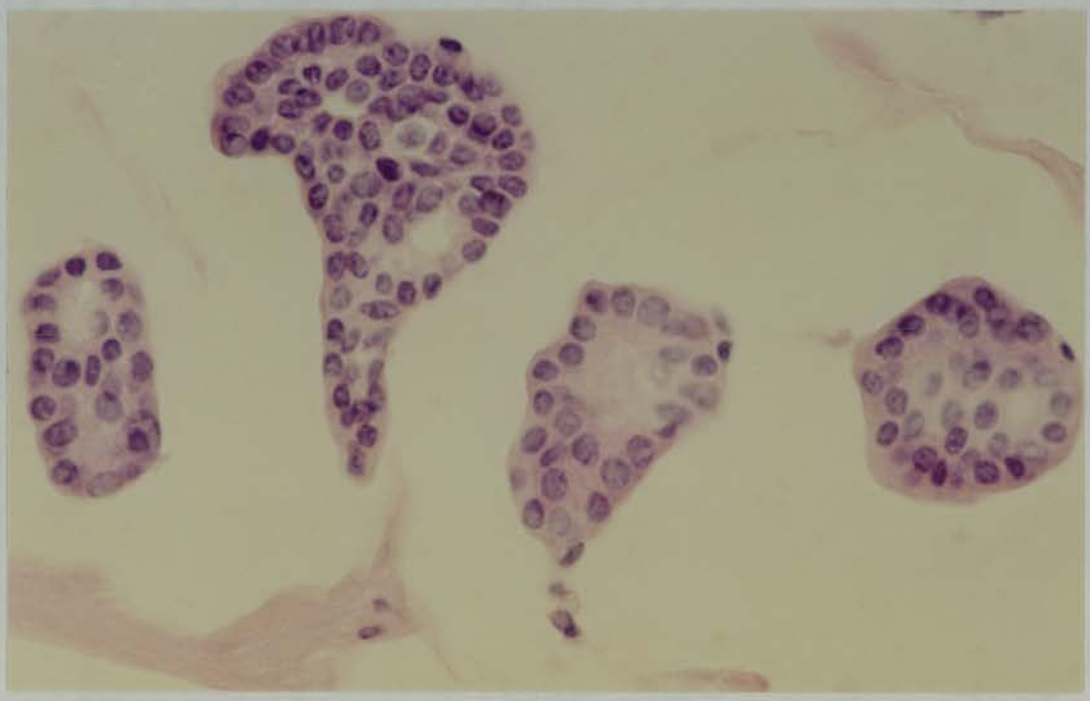


Fig. 157.

TC 73. Anaplastic carcinoma. 13 days culture. Note the granularity of the cytoplasm and similarity to the others illustrated.

Phase contrast. Original magnification x 150

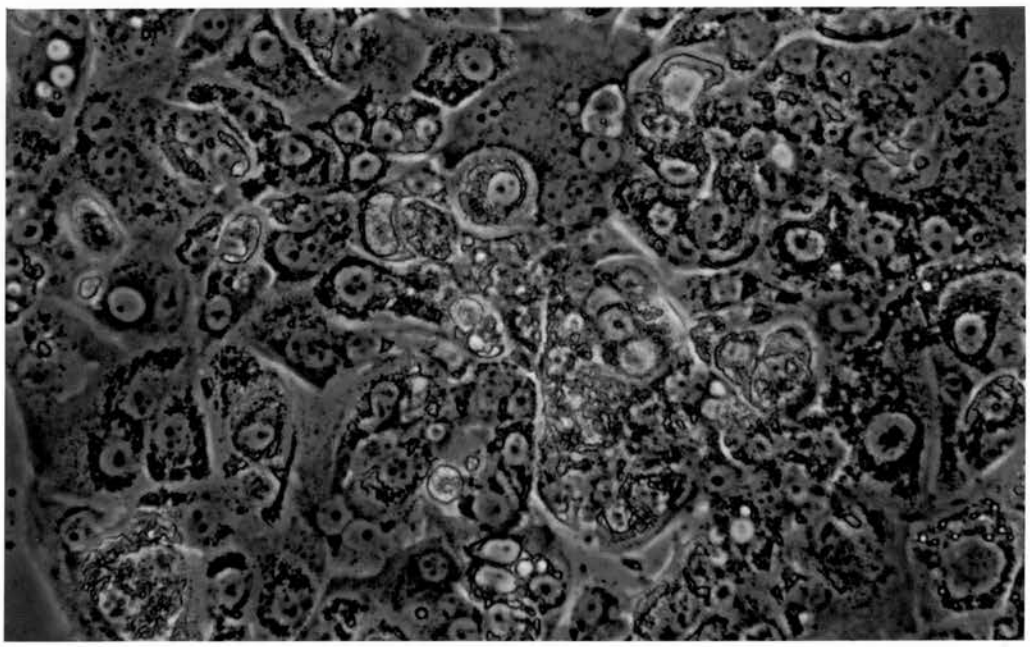


Fig. 158.

TC 85. Anaplastic carcinoma with a large cell type. 2 days culture.

Phase contrast. Original magnification x 150

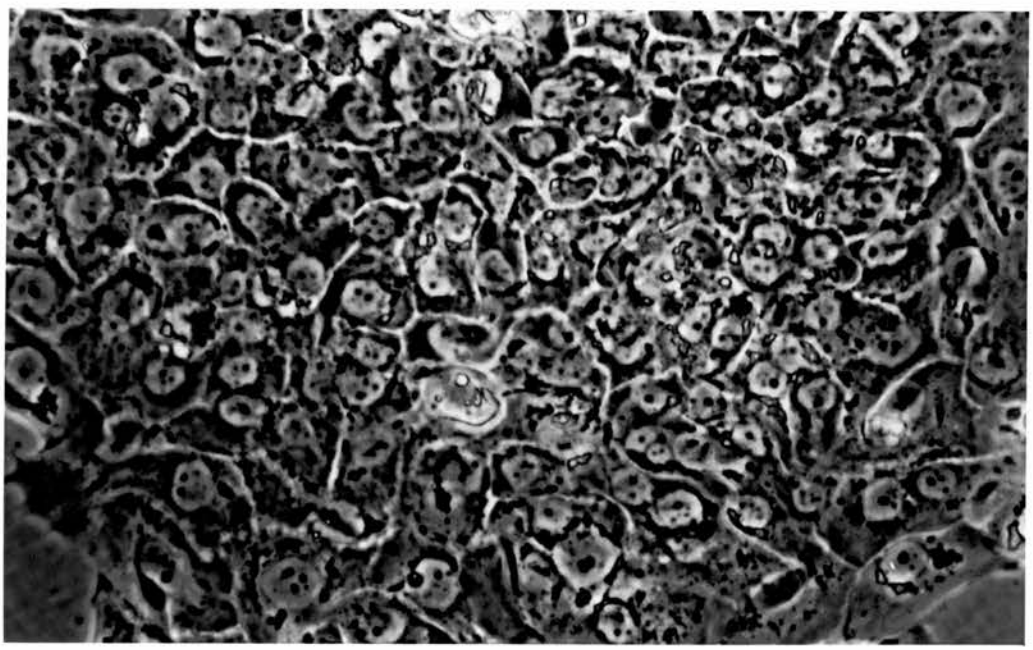


Fig. 159.

TC 38. Male, encephaloid mammary carcinoma. 4 days culture.
Typical field showing nature of apocrine cells. Phase contrast.

Original magnification x 150

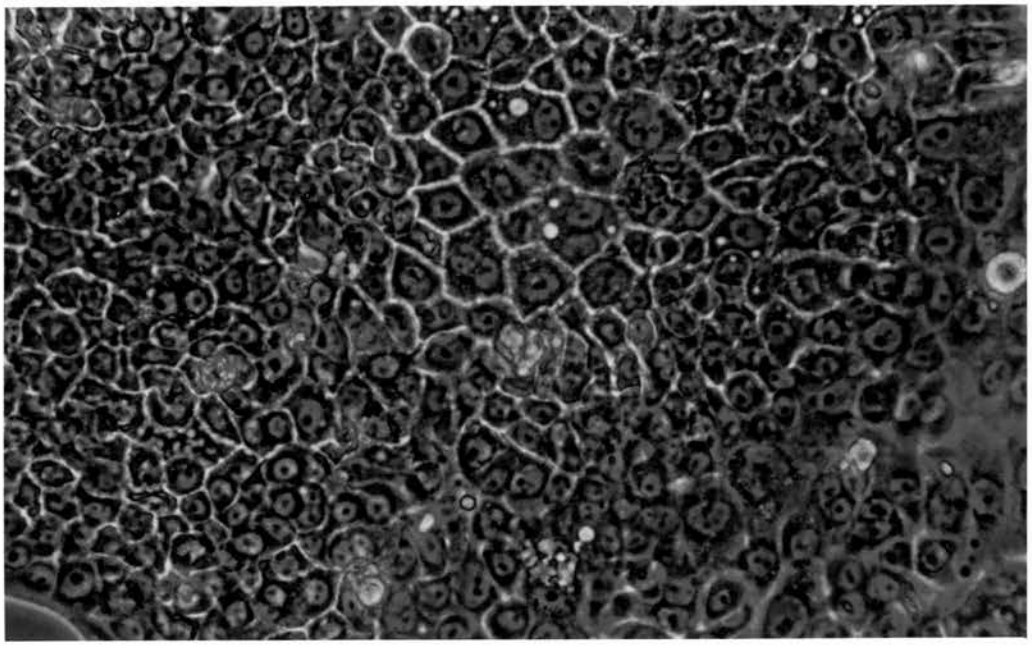
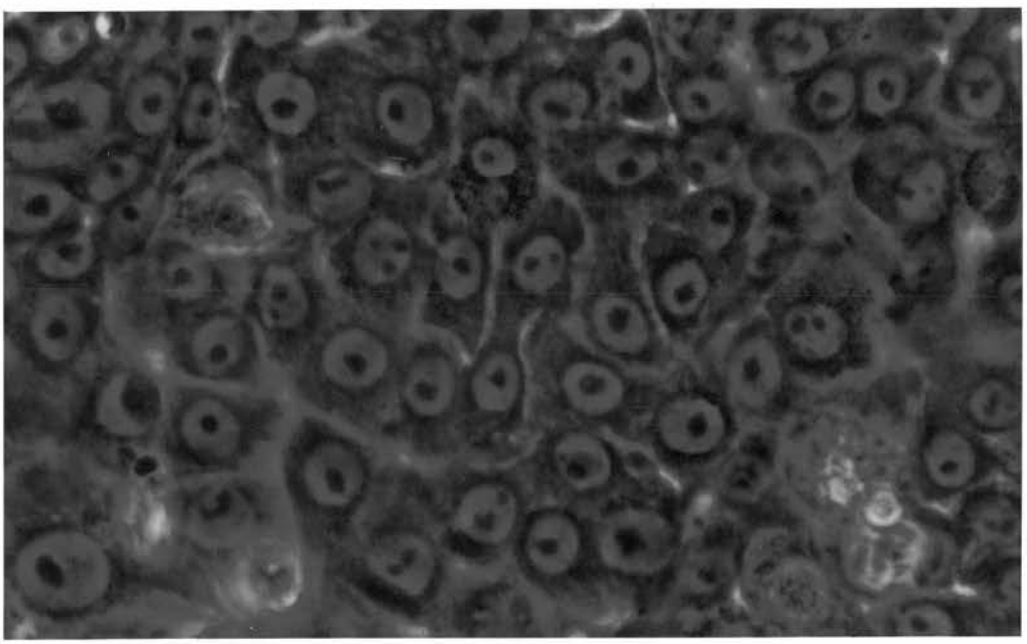


Fig. 160.

Detailed view of apocrine cells in Fig. 159. Phase contrast.

Original magnification X 300



ii) The Occurrence of Cellular "Spheres"

The occurrence of balls of viable cells floating in the medium above cultures of mammary tumours was relatively common and this type of structure was seen in 15/74 of the tissue cultures studied. The majority (14/15) occurred in cultures of tumours of low elastotic index. Cellular spheres were seen in one culture from a highly elastotic tumour (TC 68). In this series they were never identified in cultures of non-neoplastic tissue.

The spheres were sometimes present from the initiation of the culture and presumably were "spilled out" intact when the tumour was sliced during preparation but often they arose by detachment of groups of cells from the main body of the culture. The formation of cell spheres in cultures of mammary tissue has been reported previously by Hallowes et al[73]; their association with the apocrine variant of mammary carcinoma was not, however, recognised.

The cell balls occurred in a variety of shapes and sizes:-

Fig. 161. TC 68. Highly elastotic anaplastic carcinoma. 27 days culture.

Phase contrast. Original magnification x 150

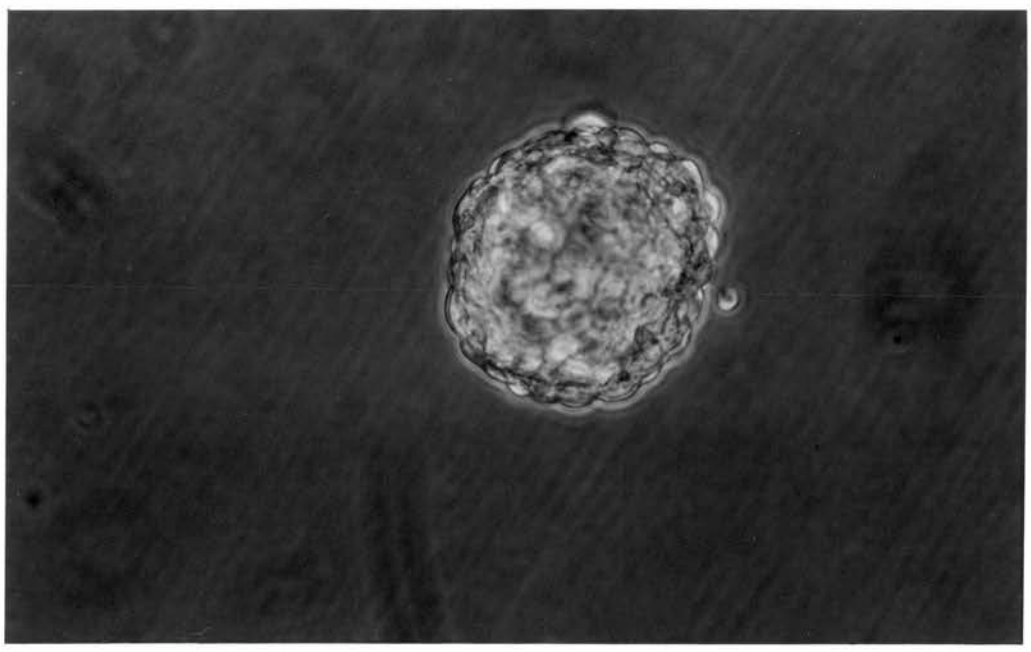


Fig. 162.

Same magnification as the preceding micrograph i.e. original magnification x 150.

Phase contrast.

TC 68. Highly elastotic anaplastic carcinoma. 27 days culture.
Same magnification as the preceding micrograph.

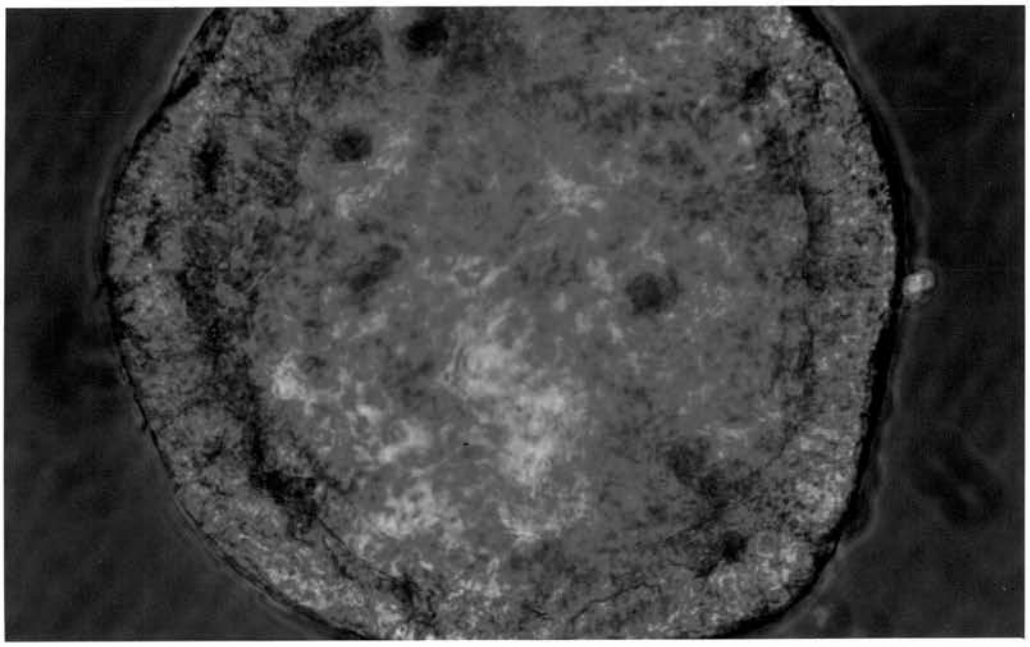


Fig. 163.
TC 26. Anaplastic mucoid carcinoma. 8 days culture.
Note the apocrine morphology of the cells in clear focus within the cellular sphere.
Phase contrast. Original magnification x 150

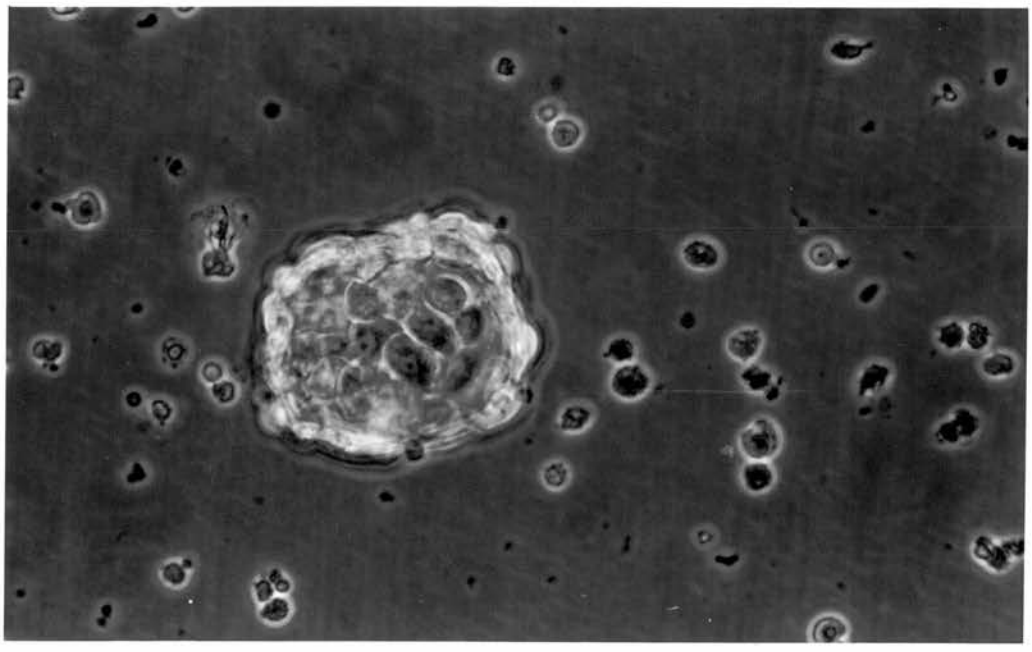


Fig. 164.

TC 38. Male encephaloid carcinoma. 3 days culture, showing part of a typical epithelial island together with a cellular sphere.

Phase contrast. Original magnification x 150

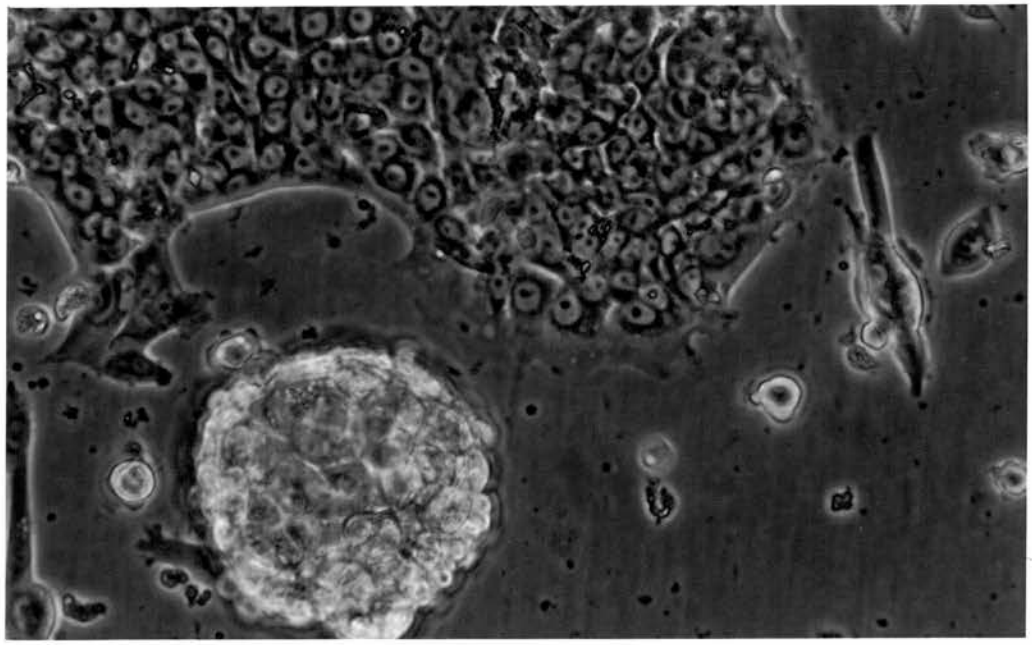
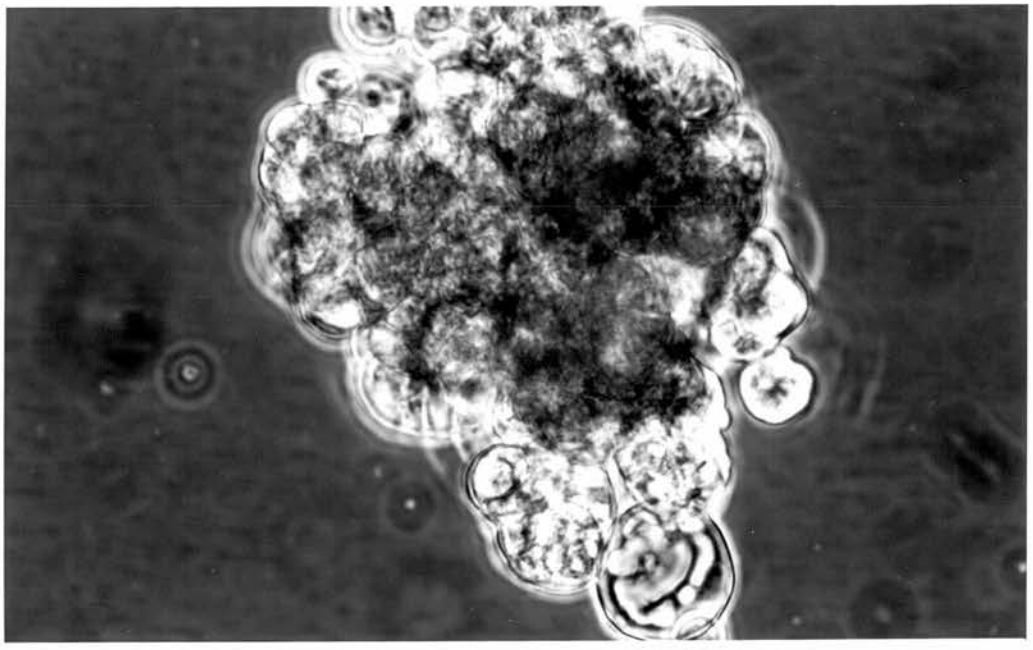


Fig. 165.

TC 74. Anaplastic carcinoma with tracts of necrosis. 12 days culture. The cell aggregates which formed in this culture were less "organised" than others.

Phase contrast. Original magnification x 150



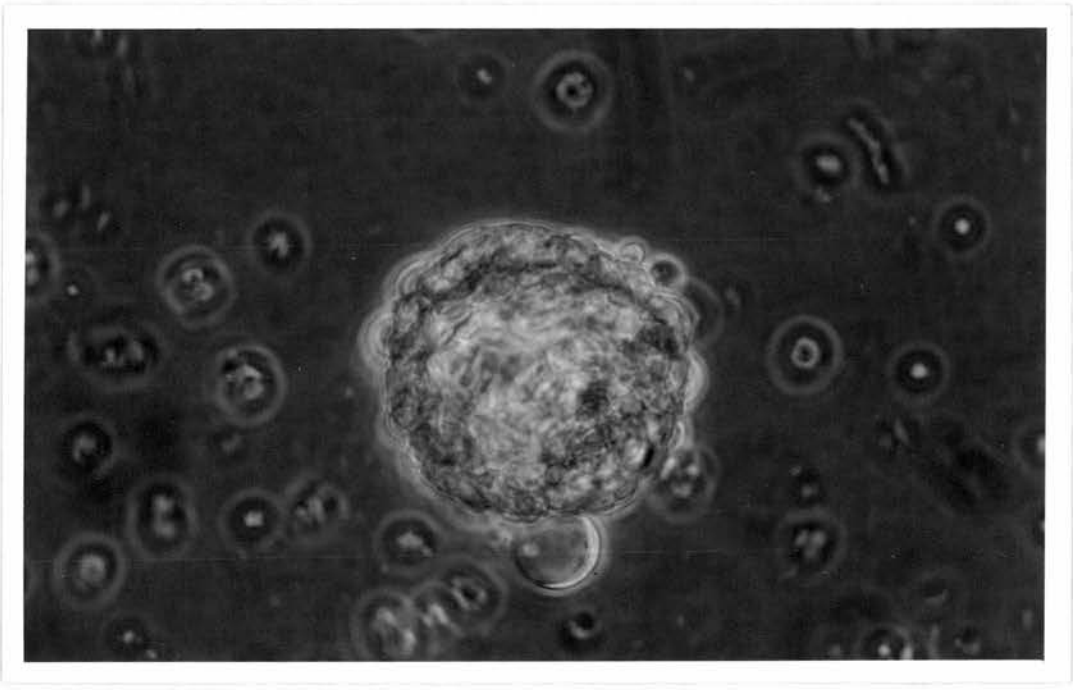
Figs. 166 and 167.

Cellular spheres demonstrating apocrine secretion.

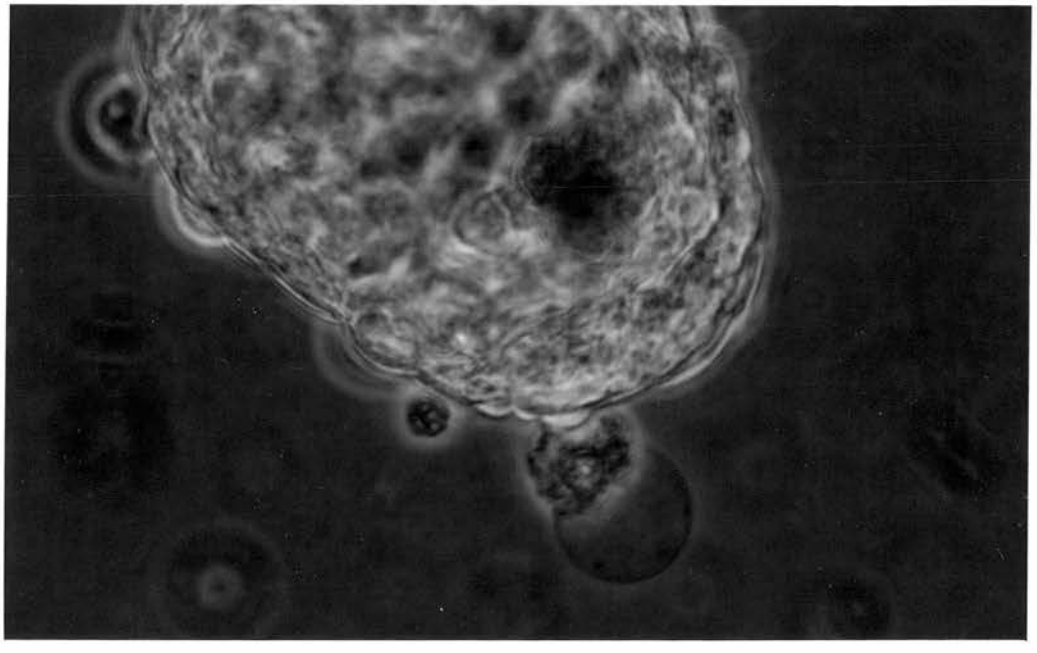
Close examination of the cell spheres revealed that they were extruding something from their surface. The belief that the component cells were of apocrine type thus received some support from their behaviour.

TC 68. Highly elastotic anaplastic carcinoma. 29 days culture.

Phase contrast. Original magnification X 150



Phase contrast. Original magnification X 300



Histological preparations were made of the cell spheres by centrifugation of the culture medium and the preparation of an agar block (pp 34). Cross sections of typical cell aggregates showed them to be composed of cells of typical apocrine morphology.

Fig. 168.
TC 72. Anaplastic carcinoma, moderate stromal component. Haematoxylin, phloxine and saffron.

Original magnification X 72

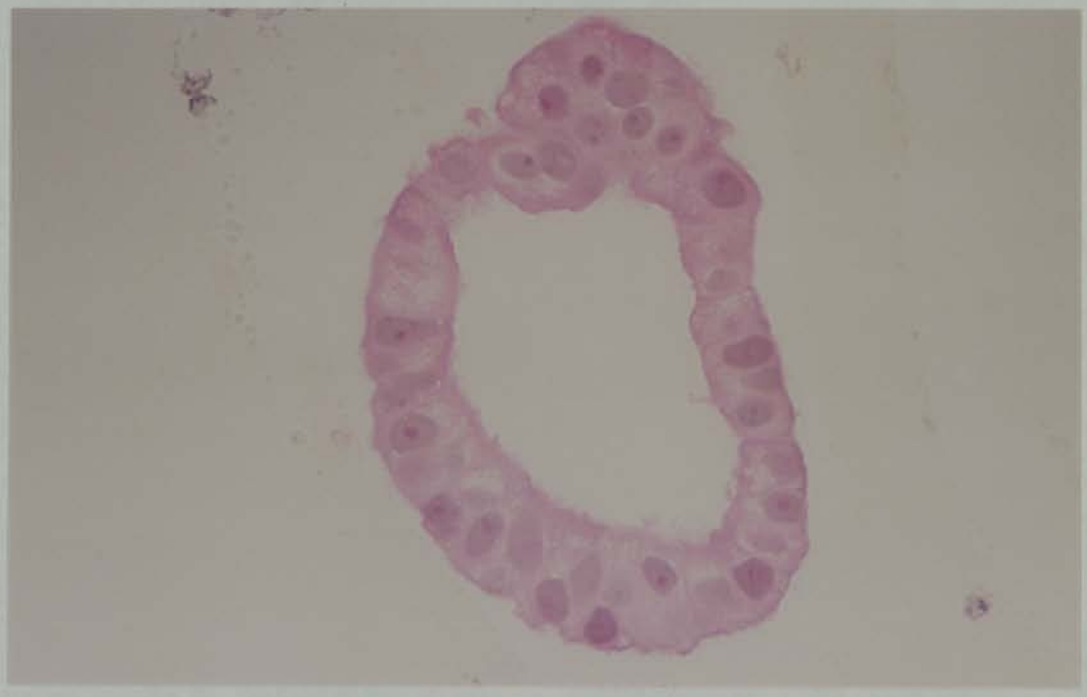


Fig. 169. Original magnification X 144

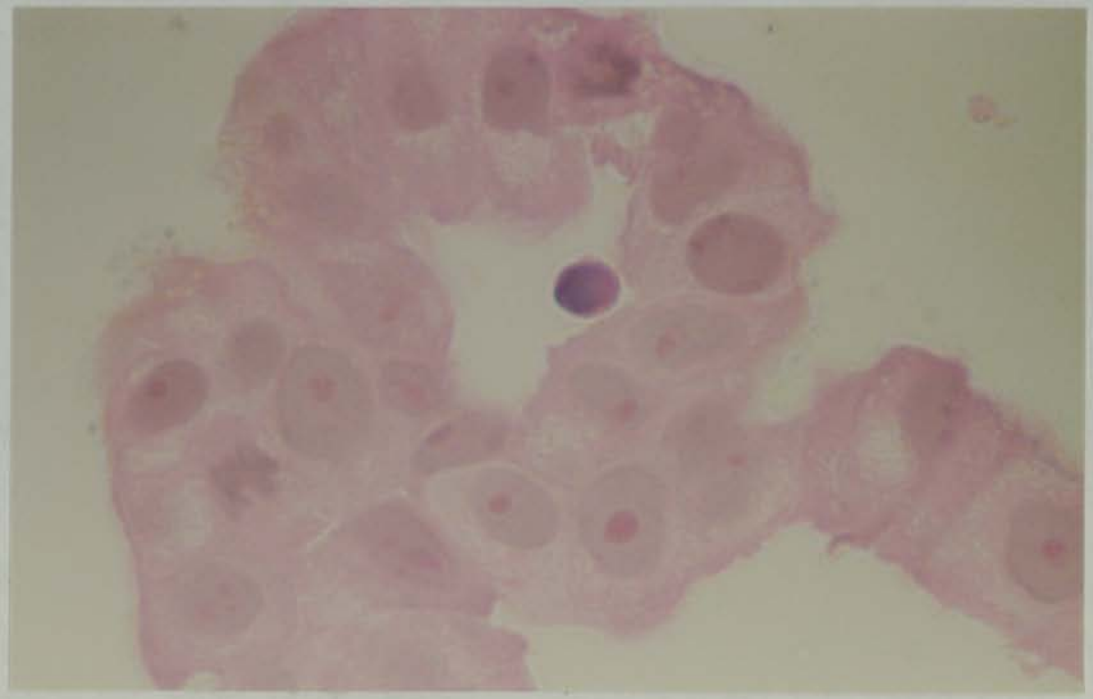
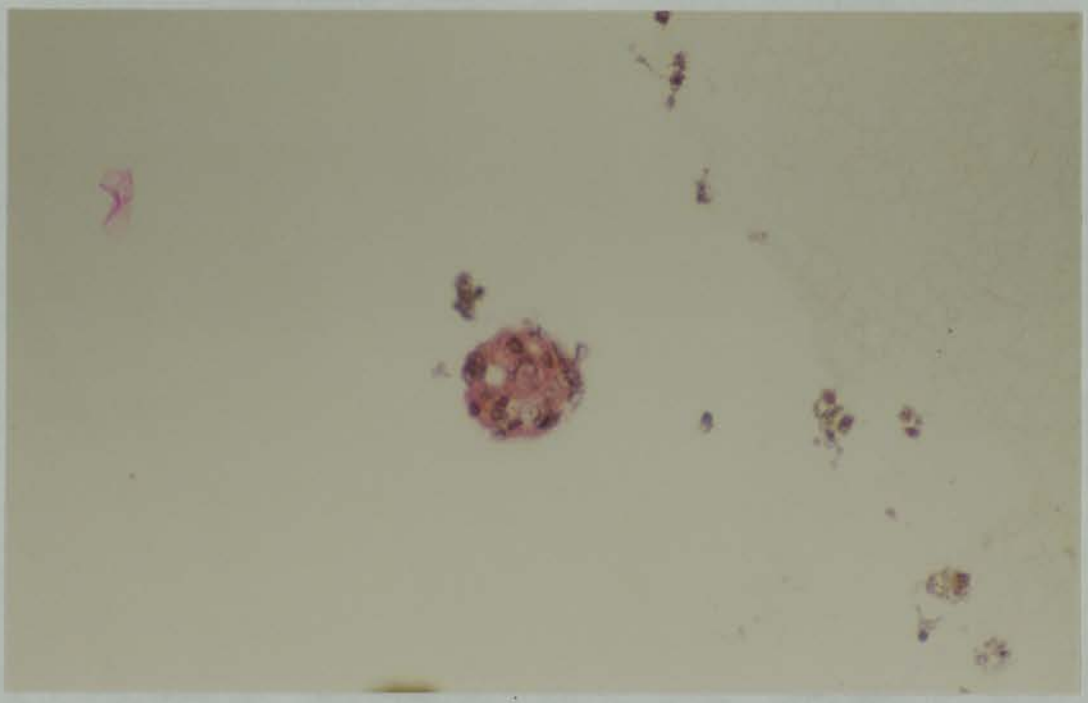


Fig. 170.

TC 48. Mucoid carcinoma plus fibrocystic disease.

Cellular sphere. Original magnification X 72



In Section II it was demonstrated that cell aggregates of this type often contained deposits of pericellular reticulin, as shown by positive staining with Gordon and Sweets' silver impregnation method.

Fig. 171.

TC 72. Anaplastic carcinoma, moderate stromal component. Cell aggregate embedded in agar block and sectioned. Gordon and Sweets' silver impregnation technique for reticulin/tartrazine. Original magnification X 144

There was also evidence, in at least one instance, that the cell spheres contained mucopolysaccharide, a feature noted often in cells of apocrine type.[124]

Fig. 172.

TC 38. Male, encephaloid breast carcinoma. 3 days culture. Cells fixed with formalin "in the flask". PAS. Original magnification X 150 Note strong positive staining of cell sphere in upper part of the field.

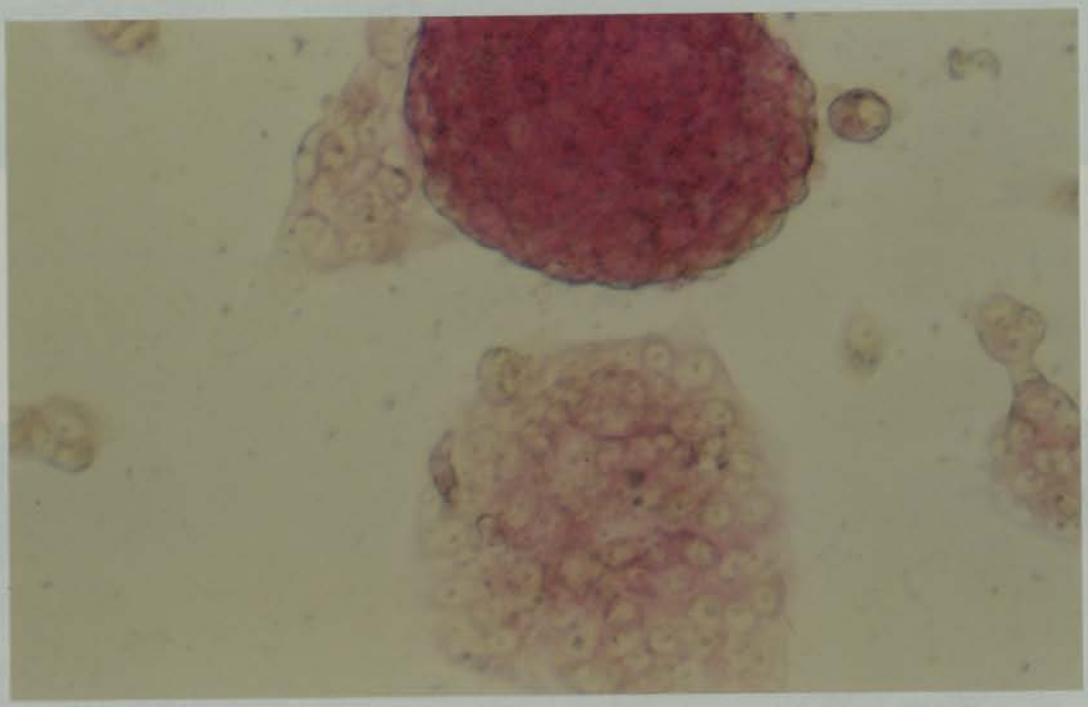


Fig. 173.

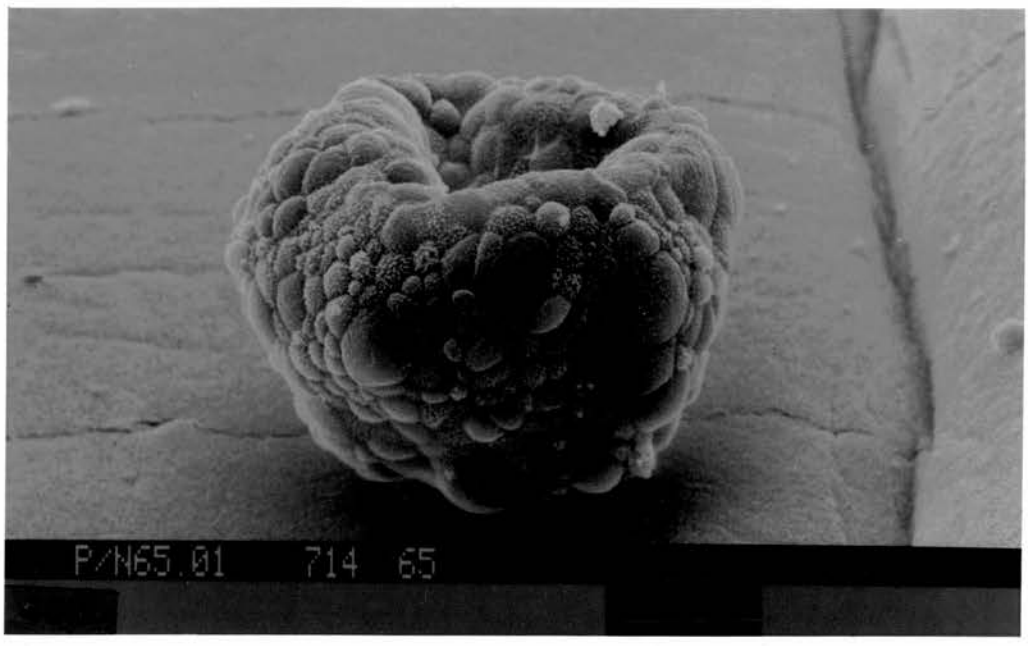
General view of Sphere I obtained from cultures of TC 68, a highly elastotic anaplastic carcinoma later shown to have an apocrine component.

Scanning electron microscopy of cellular spheres.

Two cellular spheres from cultures of TC 68, a highly elastotic anaplastic carcinoma, were sedimented onto filter paper and prepared for examination with the scanning electron microscope (pp 42).

The surface morphology of the two spheres was very varied and many types of projections could be seen on the cell surfaces. There were smooth cells, cells with short microvilli, cells with long microvilli and also cells with clumped microvilli, a feature thought by Spring-Mills and Elias[156] to be characteristic of neoplastic and pre-neoplastic mammary tissue cells.

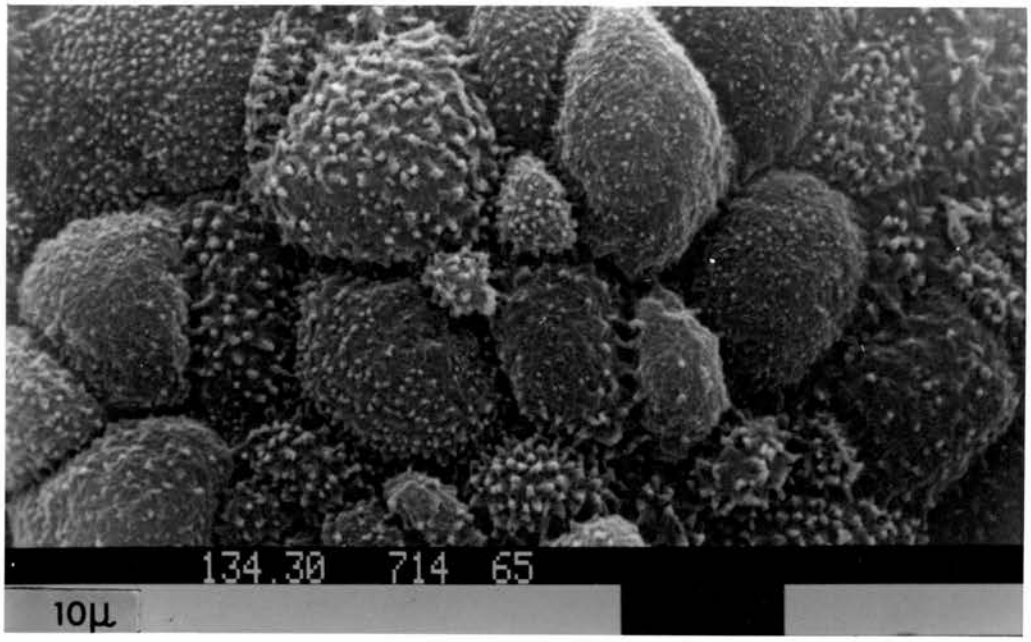
Sphere 1.



Figs. 174 and 175.

Detail of surface features of cells comprising TC 68 Sphere I. The figures show smooth cells, cells with short microvilli and cells with clumped microvilli.

Sphere 1.



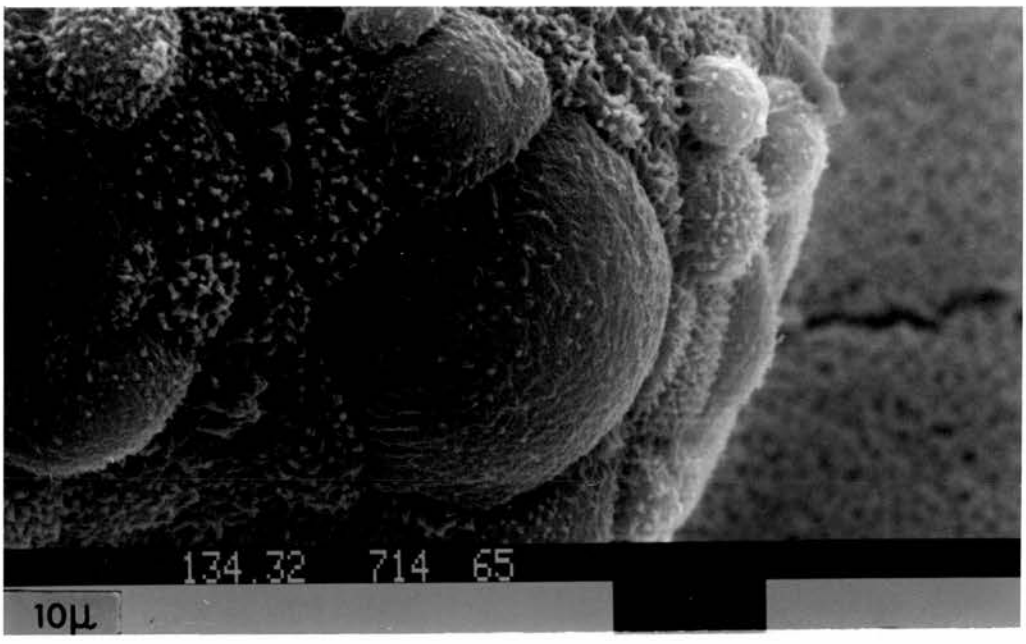


Fig. 176.

A further detailed view of the cells depicted in Fig. 173 (p 160).

Sphere 1.

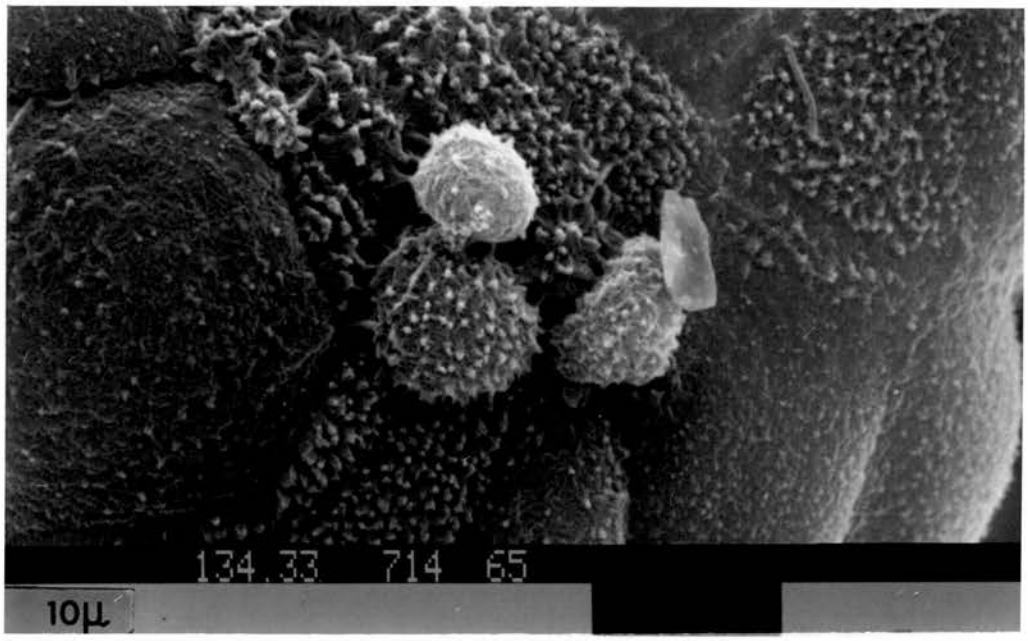


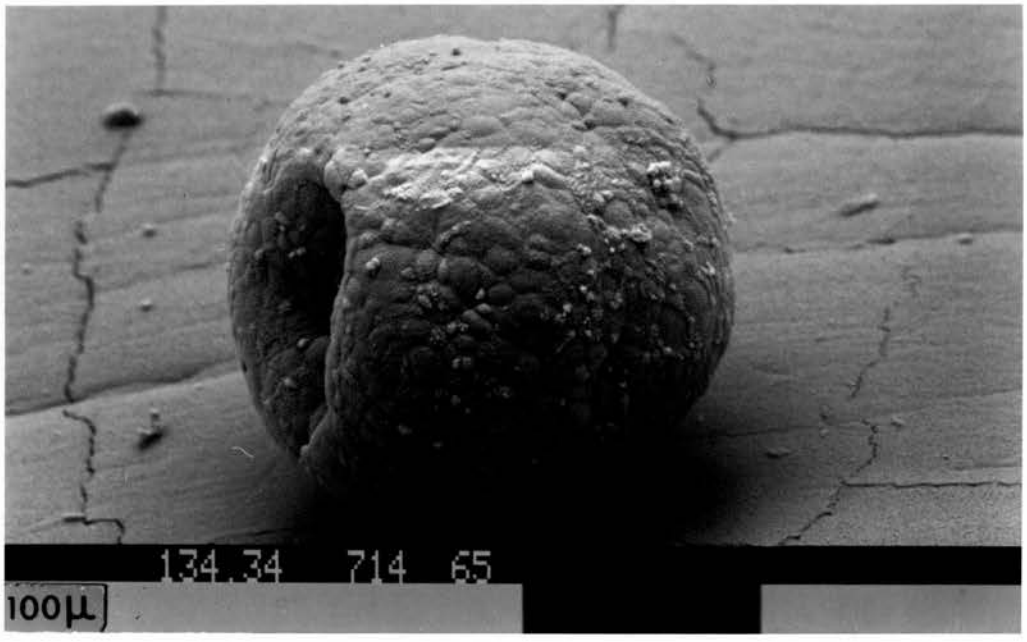
Fig. 177.

General view of Sphere II obtained from cultures of TC 68, a highly elastotic anaplastic carcinoma later shown to have an apocrine component.

Fig. 178.

Detailed view of the surface features of Sphere II.

Sphere II.



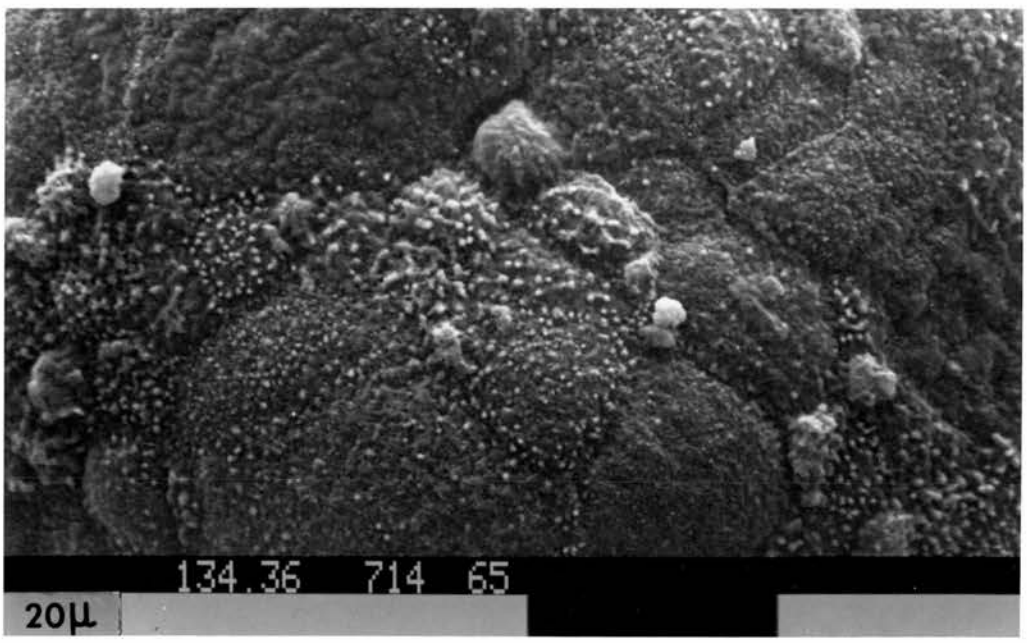
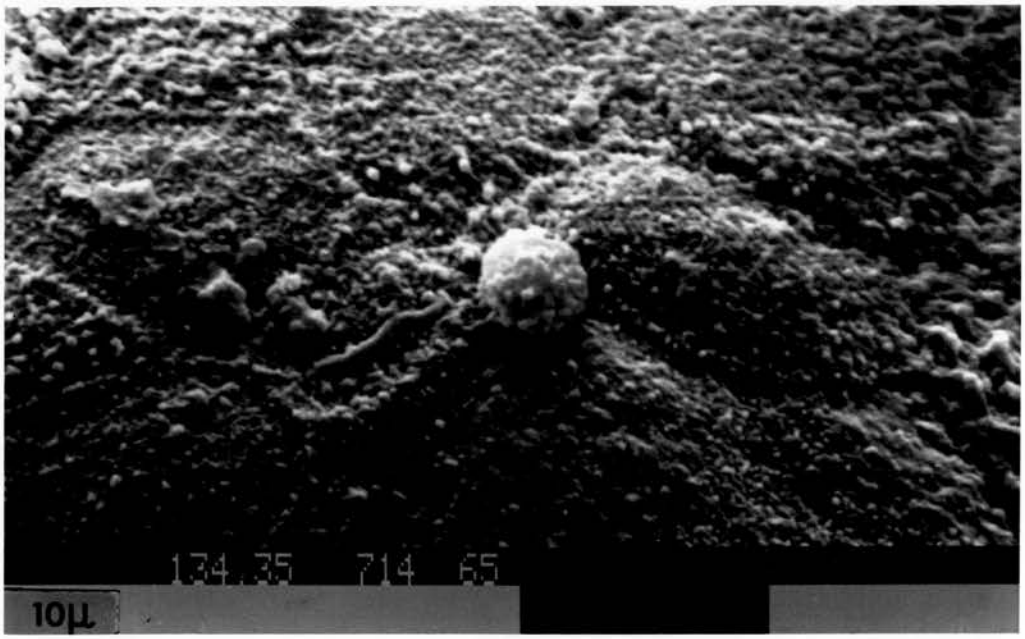


Fig. 179.
Sphere II.



The surface features in the preceding photomicrographs bear a strong resemblance to that shown as "normal" in a paper depicting the surface features of intact large mammary ducts[76]. It is possible that each sphere is composed of both normal and neoplastic cells. Sphere I was more "neoplastic" than sphere II on the basis of surface characteristics.

iii) The Occurrence of "Globules".

In addition to the morphological appearance of the apocrine cell monolayers and the occurrence of spheres of viable cells and cytoplasmic debris floating in the medium, one further feature was characteristic of cultures of this type.

A relatively common occurrence in areas of dense "fibroblast-like" growth was the appearance of large "globules" of material within the cell layers. The globules increased in number over the culture period and often seemed to be bounded by a fibrillar material.

The significance of these observations is not known but, as apocrine cells often contain PAS positive material[124] it may be surmised that the globules could be accumulations of mucopolysaccharide "trapped" within the cell layers. The premature demise of all cultures displaying this phenomenon (due to incubator malfunction) prevented further investigation.

Fig. 180.
TC 32. Anaplastic carcinoma. 15 days culture.
Globules within concentrated cell layers.
Phase contrast. Original magnification x 150

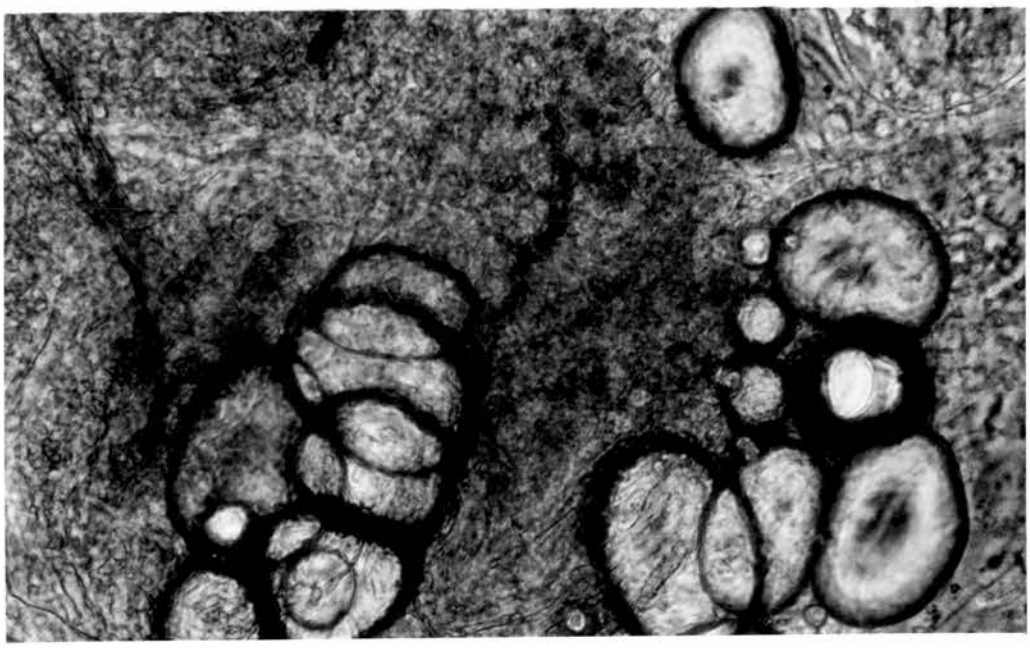


Fig. 182.

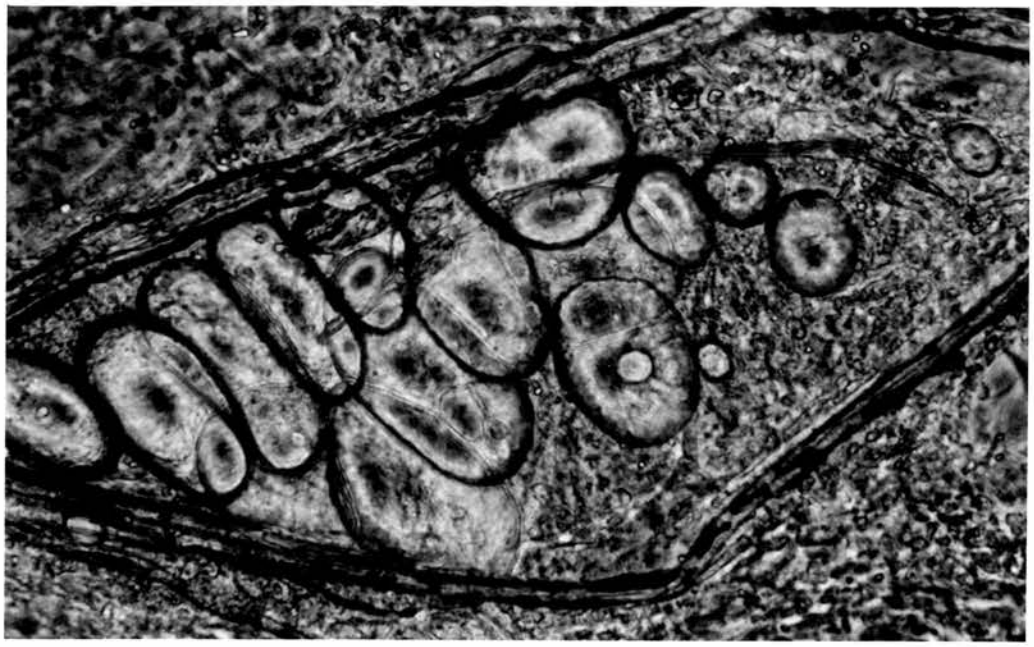
Globules deep within cell layers. Phase contrast.

Original magnification x 150

Fig. 181.

TC 32. 28 days culture. Globules bounded by "fibres".

Phase contrast. Original magnification x 150



TC 46. Anaplastic carcinoma. 9 days culture.

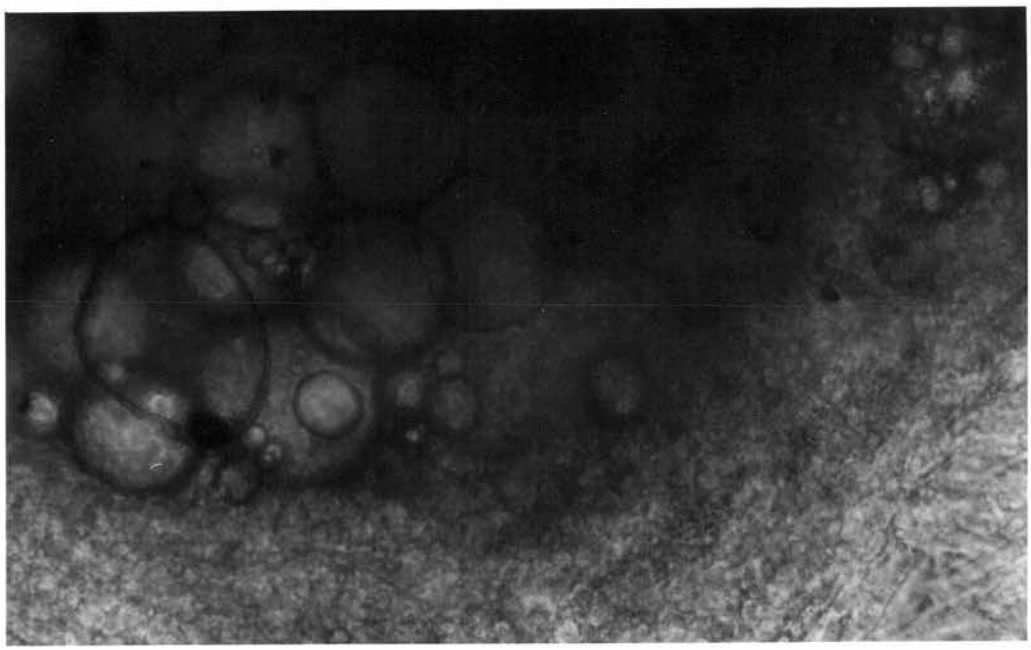


Fig. 183.

TC 46. Anaplastic carcinoma. 14 days culture. Phase contrast.

Original magnification x 150

TC 46. 14 days culture.

Note the granular cytoplasm of the cells near the globule.

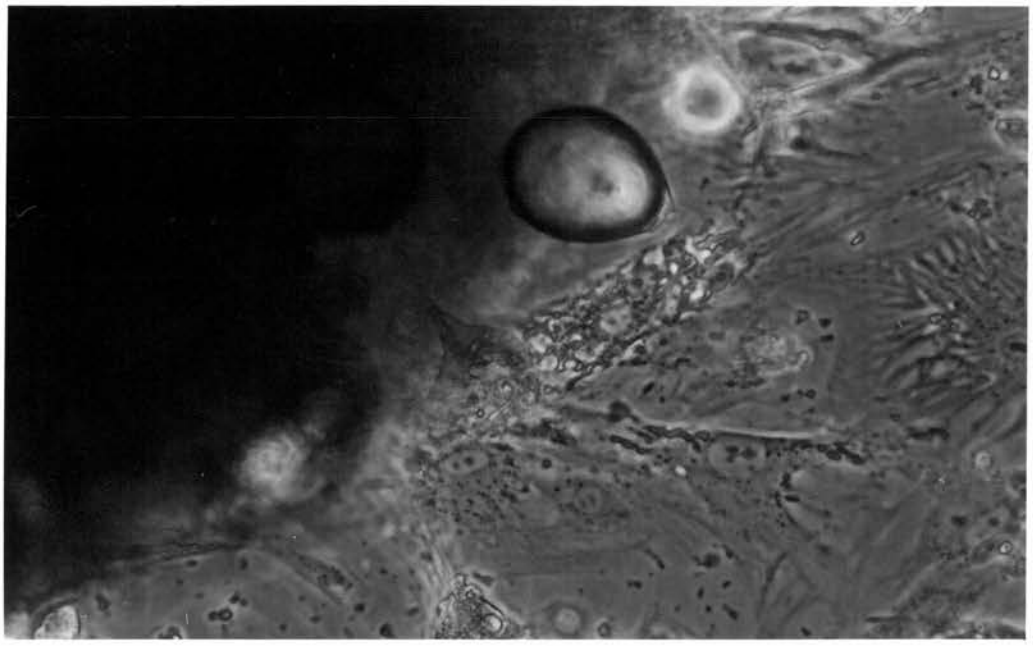
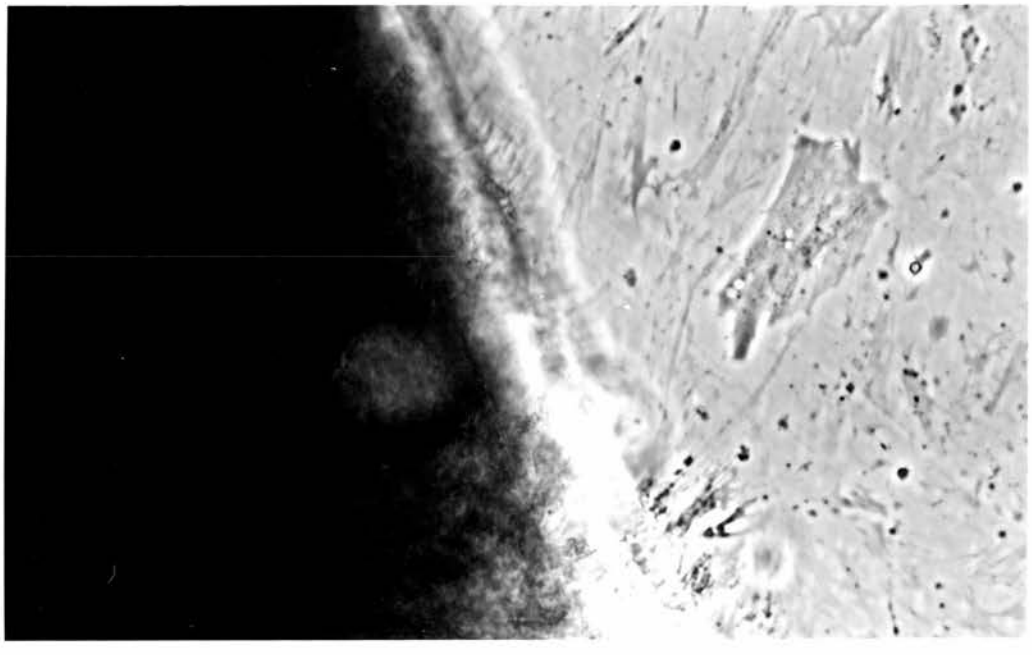


Fig. 184.

TC 83. Highly elastotic anaplastic carcinoma.

21 days culture.

Phase contrast. Original magnification x 150



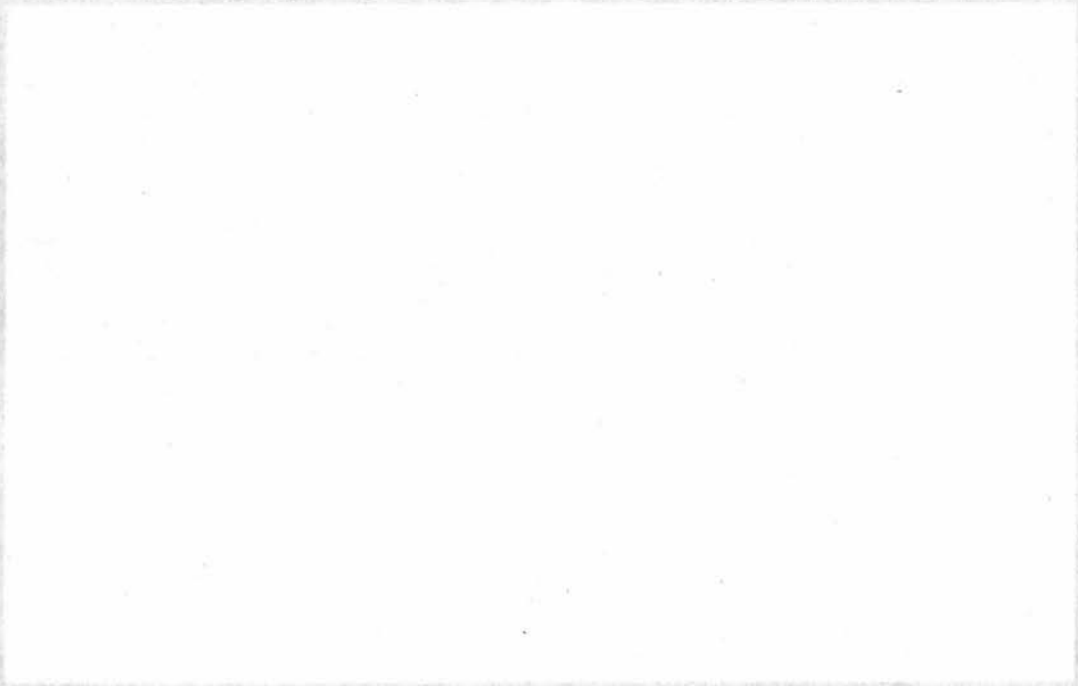
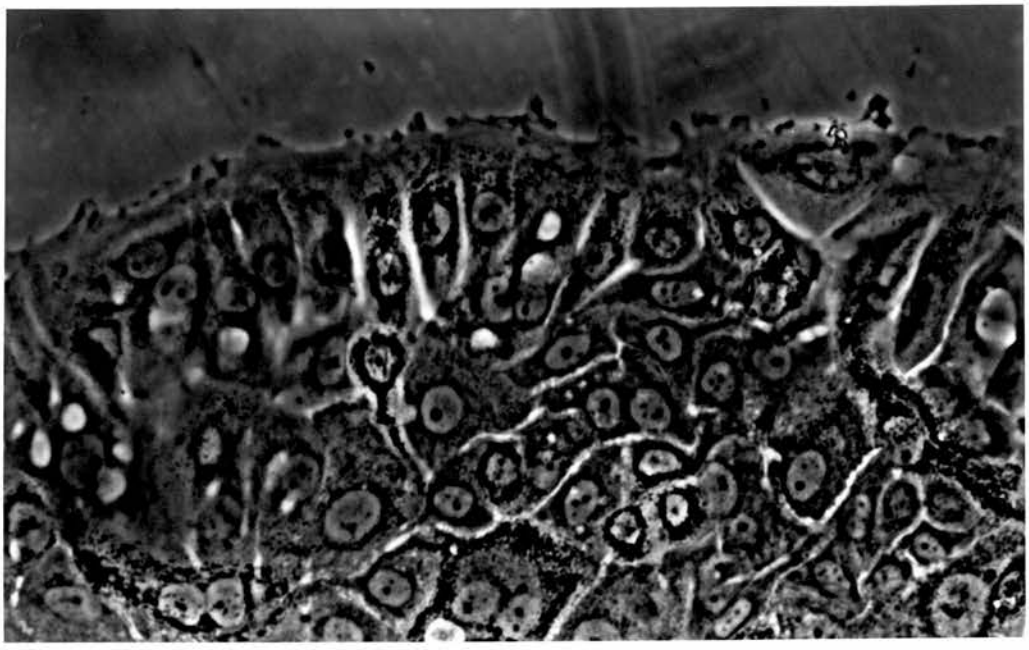


Fig. 186.
TC 76. As Fig. 185. 21 days culture showing breaking of palisade.
Phase contrast. Original magnification x 150.

The formation of a "peripheral palisade" of cells.

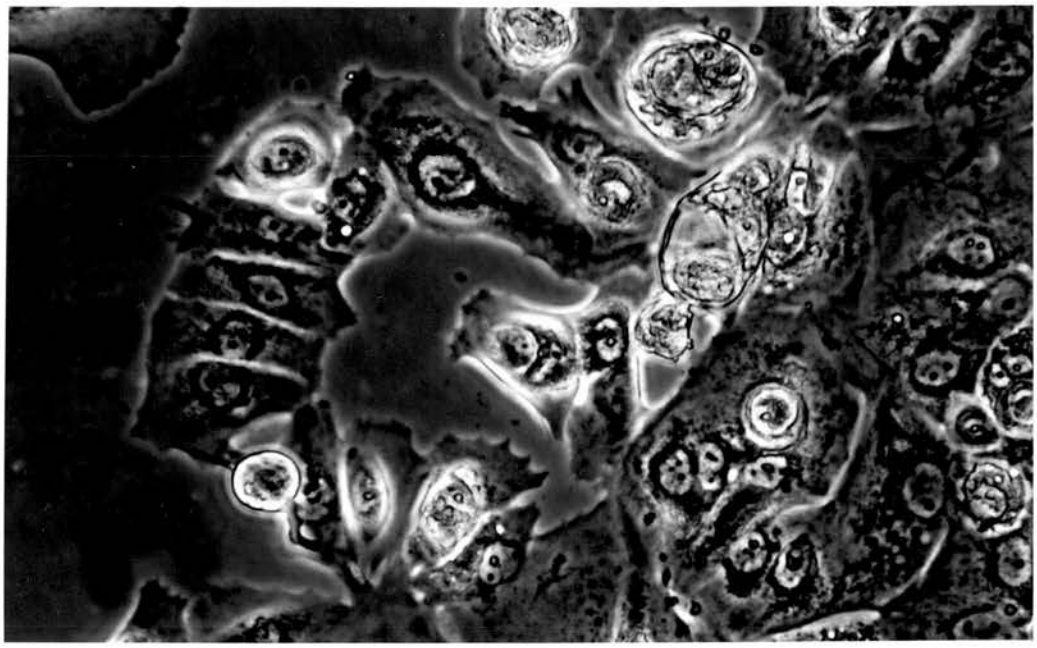
A rare, but striking occurrence was the appearance of a peripheral palisade of cells on the edge of a cell group.

Fig. 185. TC 76. Highly elastotic anaplastic carcinoma with an apocrine component. 15 days culture. Phase contrast. Original magnification x 150



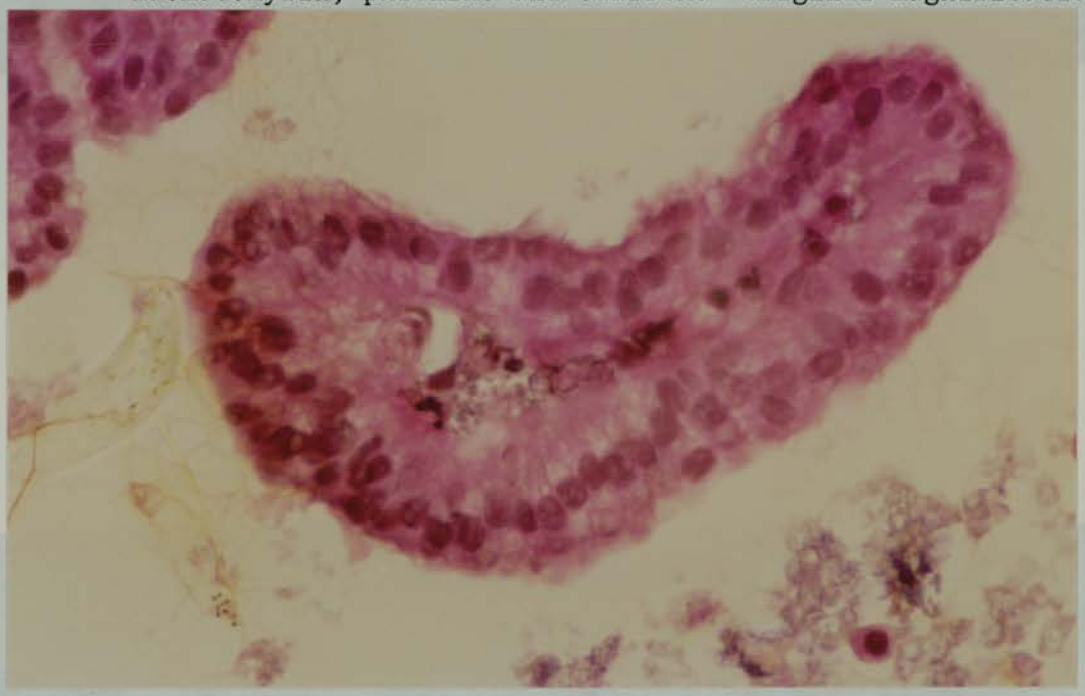
The feature was transient and gradually holes appeared in the cell monolayers as cells acquired a refringent "coat", detached from the surface and floated in the medium.

TC 76. 21 days culture.



Peripheral palisading was also seen in the walls of the floating balls of cells often found in cultures of apocrine cell type.

Fig. 187. TC 72. Cell aggregate from culture of an anaplastic carcinoma with an apocrine component. Agar embedded. Haematoxylin, phloxine and saffron. Original magnification X 72



As noted in Section II (p 116), pericellular reticulin was a feature of cell aggregates such as the one illustrated. It is likely, therefore, that the refringent areas noted in the peripherally palisaded cell monolayers were, in fact, reticulin deposits.

Fig. 188.

TC 76. Highly elastotic anaplastic carcinoma with an apocrine component. 7 days culture. General view of cytoplasmic excrescences as depicted in Fig. 44 (p 74).

Fig. 189.

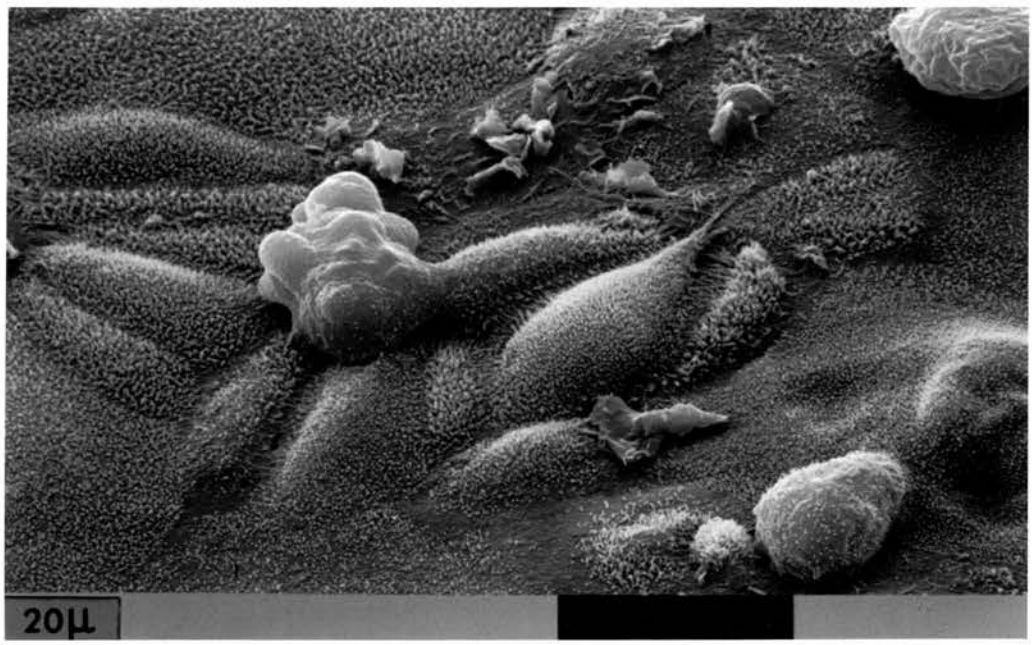
Detailed view of cell with blebs as depicted in Fig. 40 (p 72).

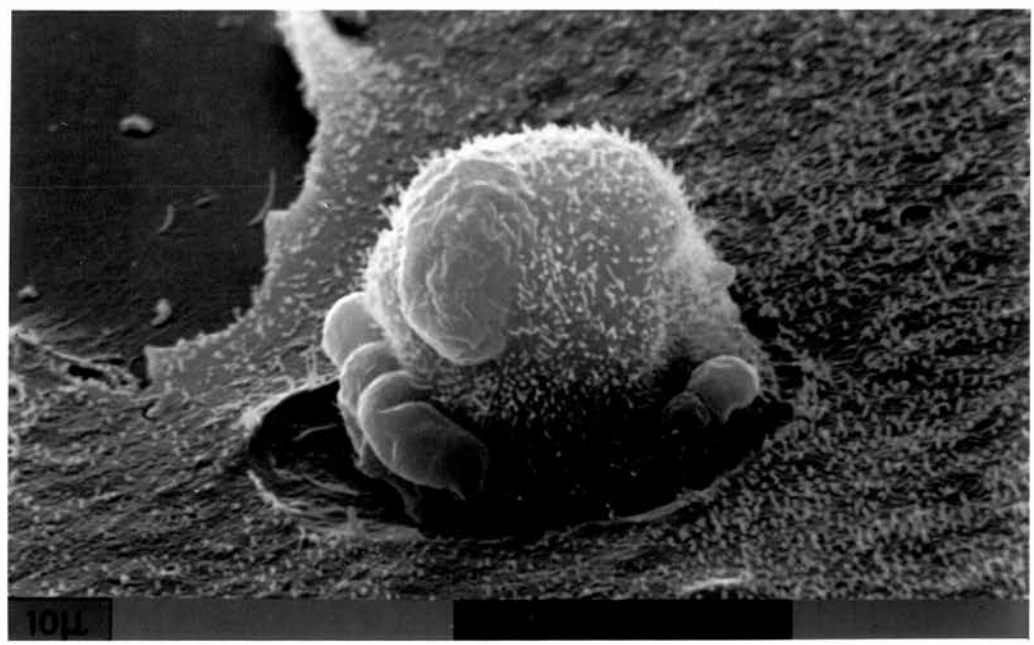
Scanning electron microscopy of cells of apocrine type.

One cell culture (TC 76), originally reported in the context of its status as a highly elastotic tumour (pp 72), was found on subsequent review of the biopsy to contain a significant proportion of cells of apocrine type.

Scanning electron microscopy of cultures from this biopsy did, indeed, reveal cytoplasmic excrescences, which might be equated with a secretory activity [108] , arising from the cells.

TC 76. Highly elastotic anaplastic carcinoma. 7 days culture.





The phenomenon was originally equated with the "blebbing" characteristic of elastotic cells and elastin was identified histologically in close proximity to such cytoplasmic blebs (pp 90).

However, scanning electron micrographs of apocrine and merocrine secretion in mammary ducts, as reported by Halter, Bradbury and Mitchell[76] do bear some resemblance to the phenomena reported above. It may be, therefore, that these micrographs do depict active apocrine secretion 'in vitro' and the possibility that apocrine secretion and elastin production may be connected is suggested.

The occurrence of the various cultural characteristics of the apocrine variant of mammary carcinoma.

Positive identification of cells of apocrine morphology, together with subsequent review of the biopsy material and histological confirmation was made in 24 cultures of neoplastic tissue.

TABLE I

No:	Original Diagnosis	Granular cells	Spheres	Globules
24	Intraduct carcinoma with considerable invasion	+	-	-
26	Anaplastic, mucoid carcinoma	+	+	-
30	Anaplastic carcinoma, moderate elastosis	+	-	-
32	Undifferentiated anaplastic carcinoma	+	+	+
36	Anaplastic carcinoma, moderate "stromal reaction"	-	+	-
38	Male, encephaloid carcinoma	+	+	-
46	Anaplastic carcinoma	+	+	+
47	Anaplastic carcinoma, variable stromal component	+	-	-
48	Mucoid carcinoma with fibrocystic disease	+	+	-
49	Adenocarcinoma	+	+	-
51	Anaplastic carcinoma	+	+	-
57	Anaplastic carcinoma	-	-	+
65	Highly elastotic anaplastic carcinoma	+	-	-
68	Highly elastotic anaplastic carcinoma	+	+	-
72	Anaplastic carcinoma, moderate "stromal reaction"	-	+	-
73	Anaplastic carcinoma, slight "stromal reaction"	+	+	-

No:	Original Diagnosis	Granular cells	Spheres	Globules
74	Anaplastic carcinoma with tracts of necrosis.	+	+	-
76	Highly elastotic anaplastic carcinoma.	+	-	-
81	Encephaloid carcinoma.	+	+	-
82	Highly elastotic anaplastic carcinoma.	+	-	-
83	Highly elastotic anaplastic carcinoma.	+	-	+
84	Anaplastic carcinoma, large cell type.	-	+	-
85	Anaplastic carcinoma, large cell type plus fibrocystic disease.	+	+	+
86	Highly elastotic anaplastic carcinoma.	+	-	+

Fig. 190.

Cells of apocrine type. Phase contrast. Original magnification x 150.

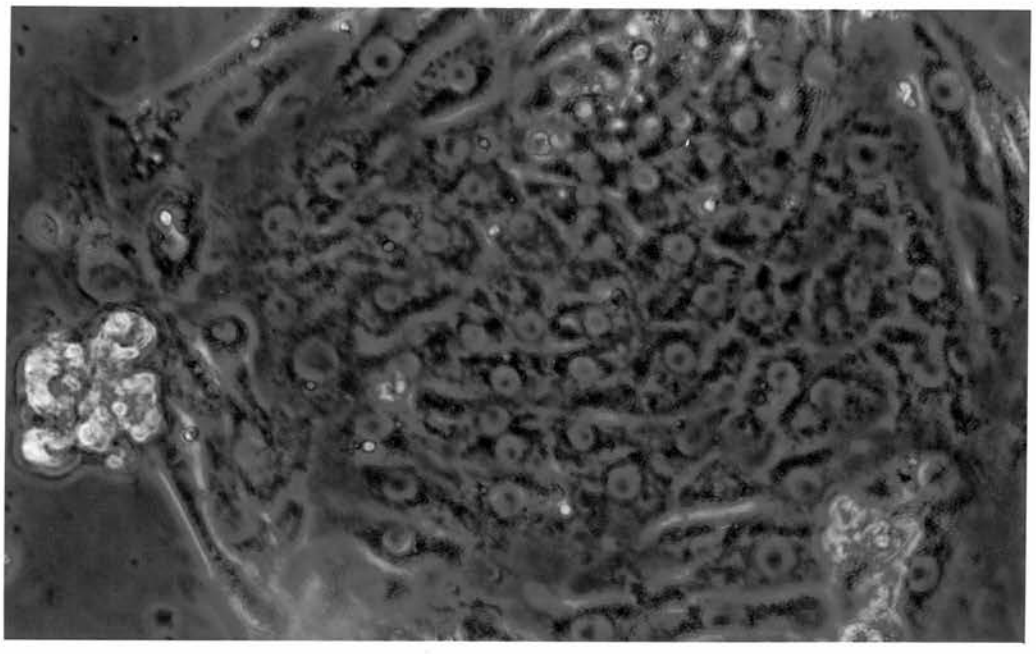
Fig. 191.

Cells of apocrine type. Phase contrast. Original magnification x 150.

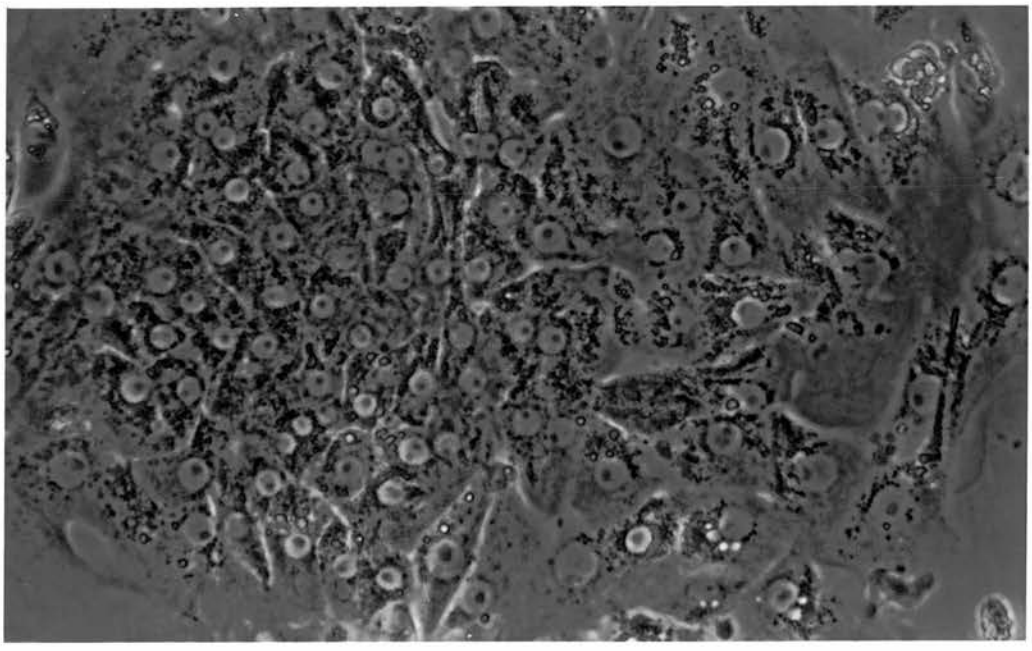
THE DEMONSTRATION OF CELLS OF APOCRINE TYPE IN NON-NEOPLASTIC BREAST TISSUE CULTURES

In addition to the occurrence of cells of apocrine type in cultures of neoplastic tissue, this type of cell was seen in cultures from one case of fibrocystic disease and one case of fibroadenosis with cyst formation.

TC 12. Fibrocystic disease with considerable adenosis. 2 days culture.



TC 19. Fibroadenosis with cyst formation. 3 days culture.



Review of the biopsies in both cases confirmed the presence of cells of apocrine type.

Fig. 192. TC 19. Fibroadenosis. Biopsy specimen. H & E.

Original magnification X 14.4

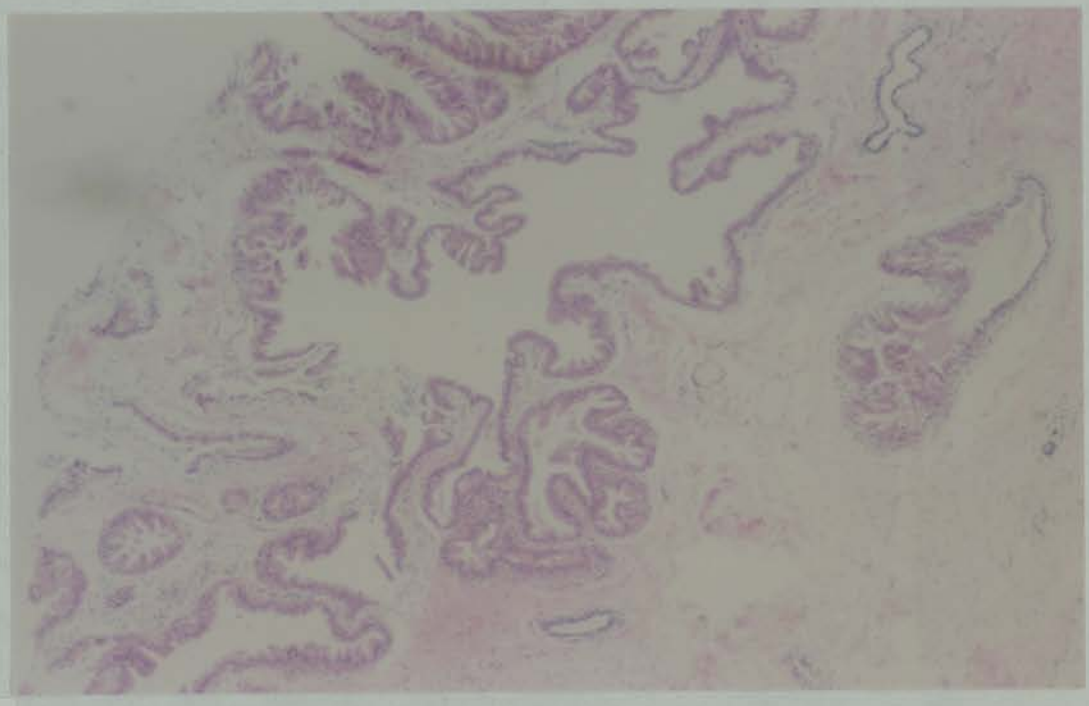


Fig. 193.

Note characteristic 'pink-cell' staining and apparent apocrine secretion into the lumen of the cyst. Original magnification X 144

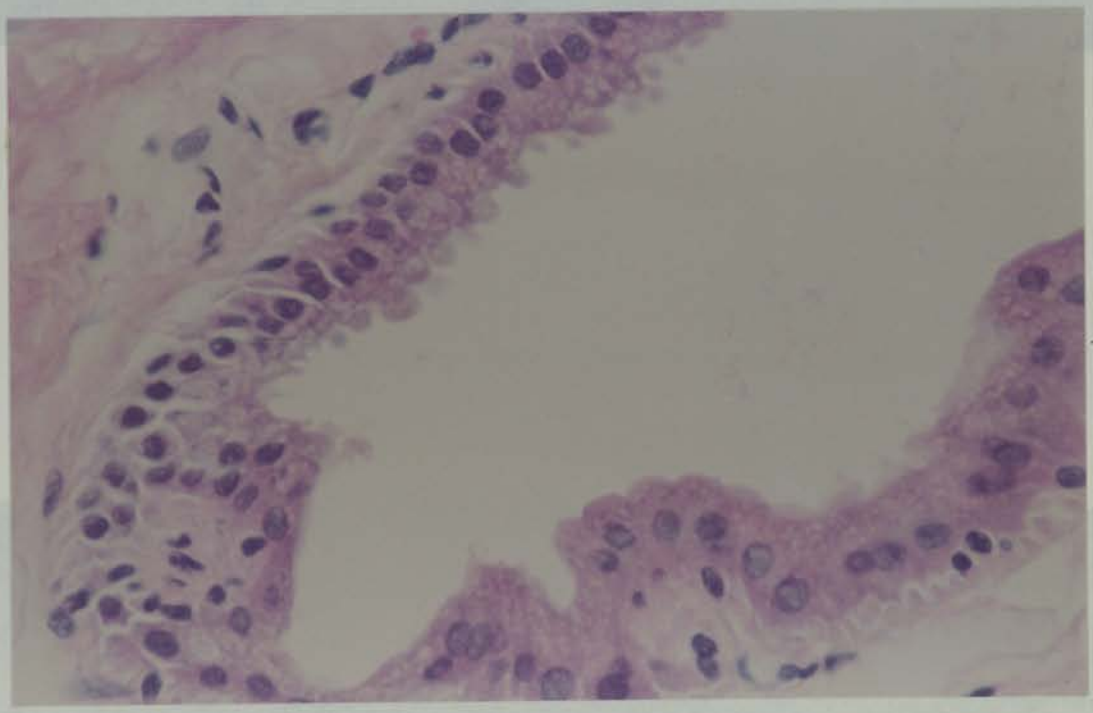


Fig. 194.

TC 30. Anaplastic carcinoma with moderate elastosis and an apocrine component. 36 days culture showing 'blebs' and vacuoles. Phase contrast.

Original magnification x 150

SECTION IV

THE OCCURRENCE, IN CULTURE, OF A PHENOMENON ANALOGOUS TO METASTASIS

Diminished mutual adhesiveness of tumour cells is one of the concepts used in explaining invasive growth in malignant tumours, based on changes observed in cell membranes[116]. The demonstration of exfoliation of both single cells and small groups of cells in the apocrine cell cultures lends support to the suggestion that diminished mutual adhesiveness is a feature of mammary carcinoma.

One further observation, made during the present study in two of the cultures of tumours of apocrine type, would seem to equate with the phenomenon of metastasis; that is migration of neoplastic cells to a distant site followed by an upsurge of tumour growth.

The cultural characteristics of TC 30, an anaplastic carcinoma with moderate elastosis and an apocrine component.

The epithelial cells which grew from TC 30 were of characteristic apocrine morphology with large, pale nuclei and coarsely granular cytoplasm. During the initial phase of growth - 1 month - cells could be seen detaching from the main cell pavement and floating in the medium. Cell detachment appeared to lead to the production of a vacuole. **V**

TC 30. 36 days culture.

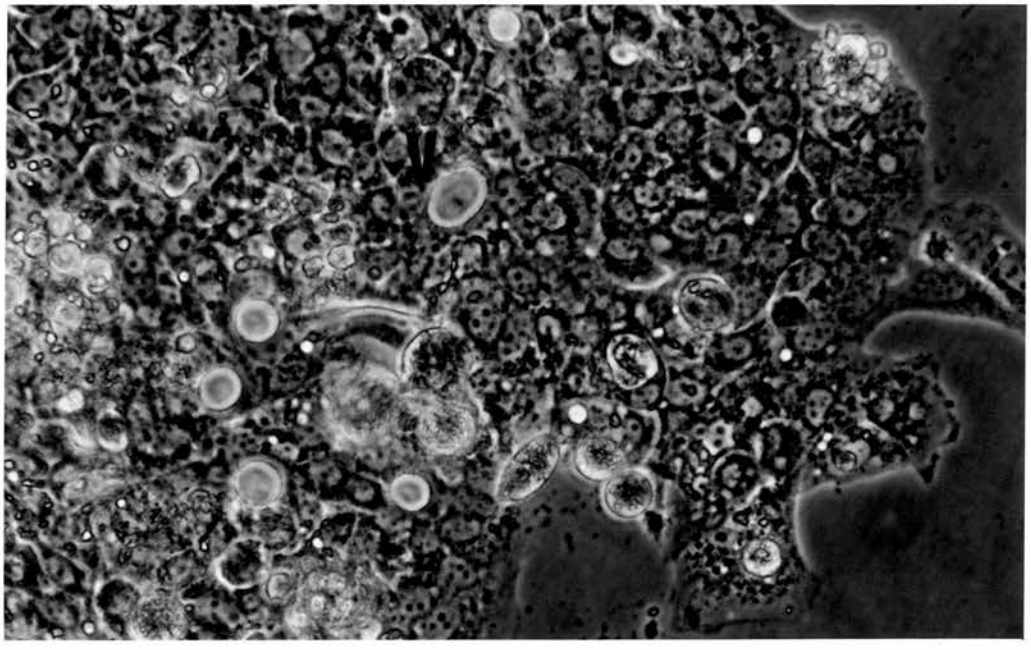


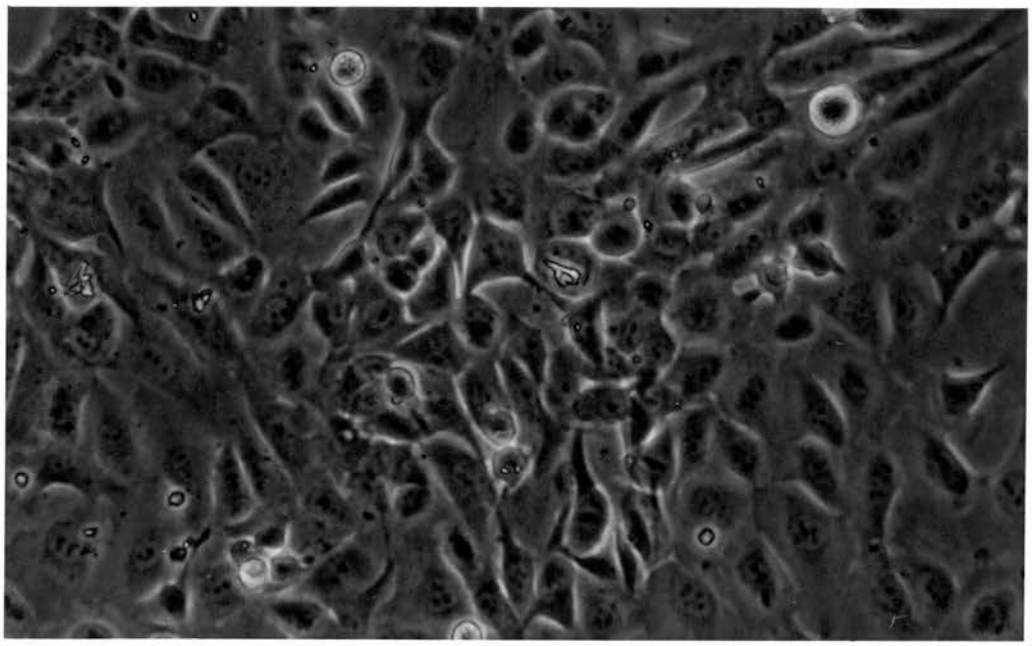
Fig. 195.

TC 30. 50 days culture. Cells of a new morphological type which appeared in one culture flask 48 days after culture initiation. Phase contrast.

Original magnification x 150

Forty eight days after culture initiation a new morphological type of cell appeared. These cells were small, had a large nuclear/cytoplasmic ratio and multiple nucleoli within each nucleus. Unlike those cells of apocrine morphology, the new cell type divided very rapidly to form a large cell pavement.

TC 30. 50 days culture.



Cell division continued until incubator malfunction terminated the culture 56 days after initiation.

TC 24. Intraduct carcinoma with considerable invasion and an apocrine component.

A similar phenomenon was observed in cultures of TC 24.

Initially islands of granular epithelial cells grew slowly and were seen to release cell contents into the medium.

Fig. 196.

TC 24. Intraduct carcinoma with considerable invasion and an apocrine component. 23 days culture. Typical field of cells. Phase contrast.

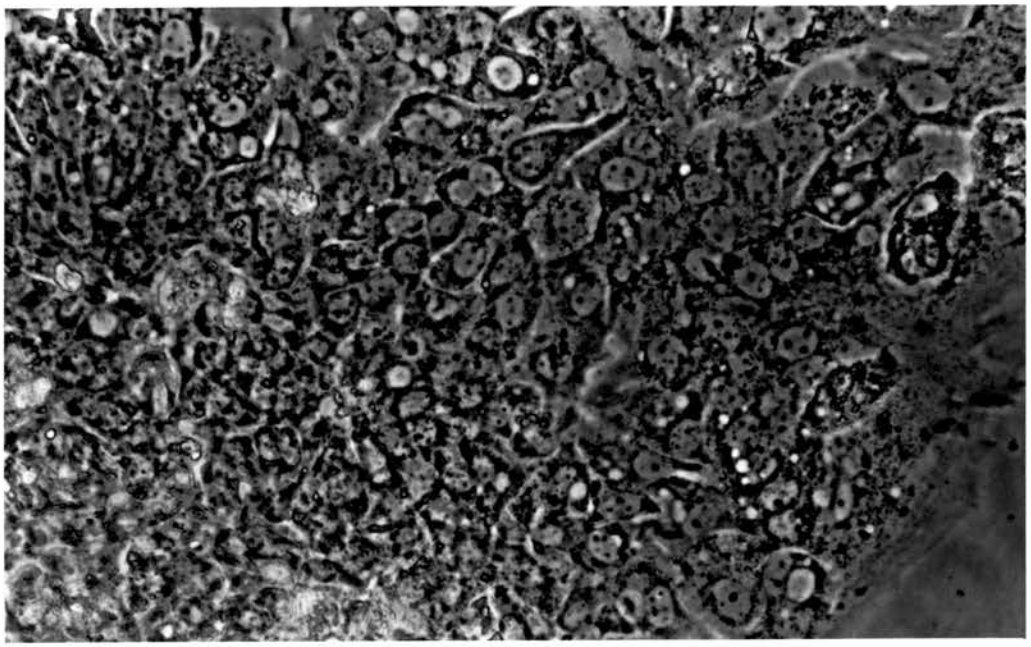
Original magnification x 150

Fig. 197.

TC 24. New cell type which arose in one flask 12 days after culture initiation. Phase contrast.

Original magnification x 150

TC 24. 23 days culture.



Twelve days following culture initiation a new type of cell appeared. These cells were very similar in appearance to those which occurred in TC 30 and, like the latter, divided rapidly to form an extensive cell pavement.

TC 24. 12 days culture.

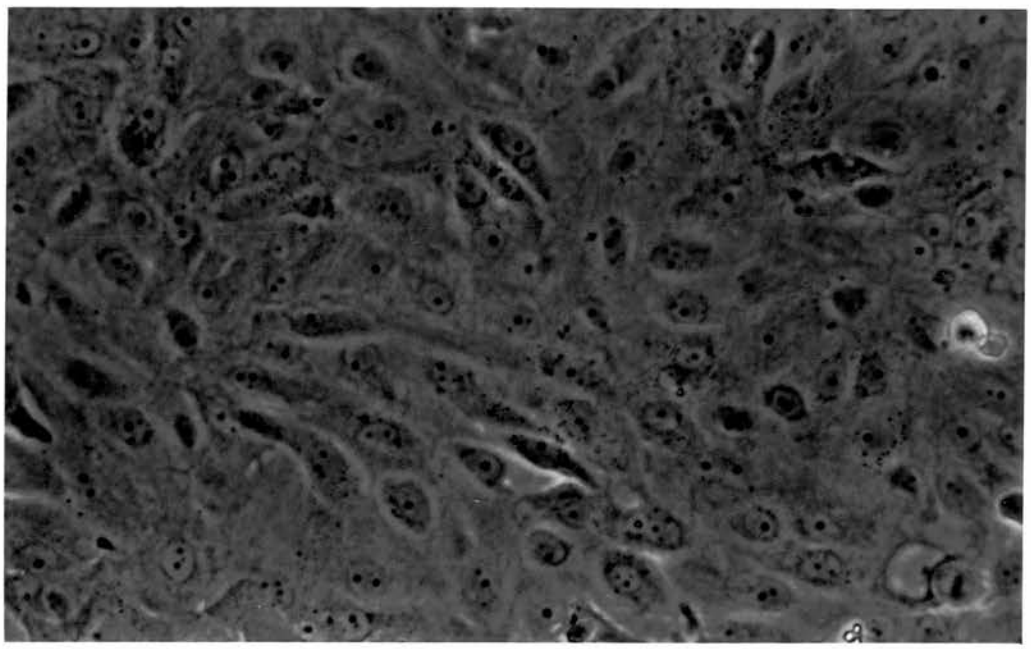
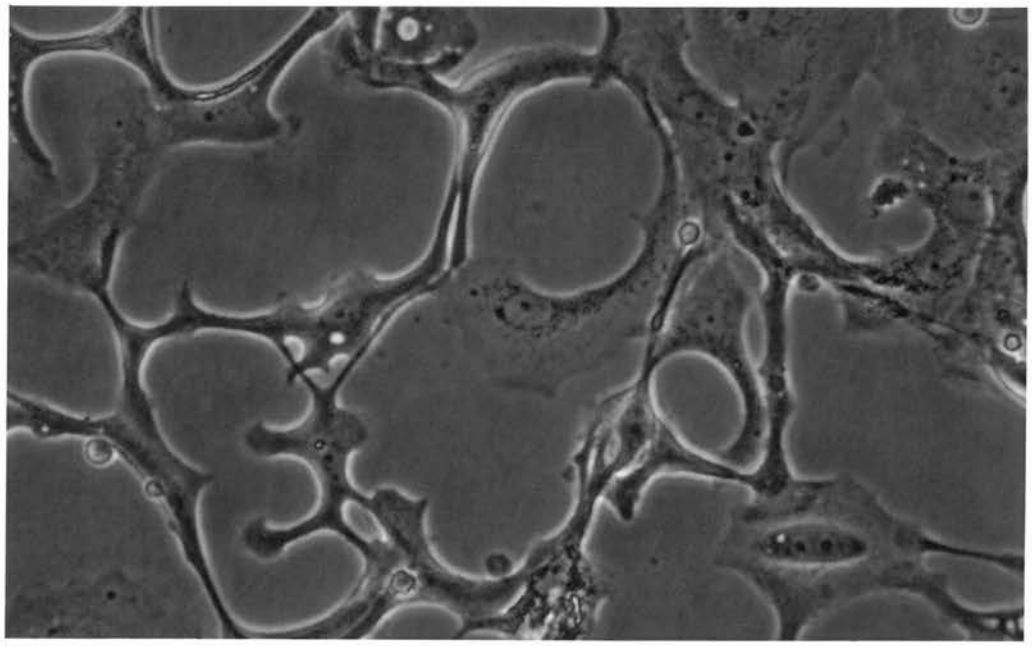


Fig. 198.

TC 24. Senescent cells exhibiting refringent haloes 41 days after culture initiation. Phase contrast. Original magnification x 150

By 23 days culture both the apocrine and the "metastatic" cell sheets had begun to develop holes in them and the islands of cells remaining acquired a "halo". These cells were finally lost into the medium and the cultures were terminated 74 days after initiation.

TC 24. 41 days culture.



The observation of an analogue of metastasis is not a new observation. Coman [33], in 1942, noted that single epithelial cells in cultures of mammary carcinoma not infrequently "broke away from the small nests and clusters (of cells) ..... Proliferation of these isolated cells sometimes formed new colonies". He went on to speculate on the possible relationship that this phenomenon might have to invasion and metastasis and also to comment on the subject of "cohesiveness of the cells".

SECTION V.

CELL LINES

During the course of the study two primary cell lines were derived from the human mammary tissue cultured. The lines were designated CTH 1 and CTH 7.

CTH 1

CTH 1 was derived from a culture of a lobular carcinoma, the first passage being made 17 days after initiation of the original culture. (TC 1) The morphology of the cells was varied. At low cellular concentration the cells were rather stellate in appearance but, as the concentration increased, the cells elongated and took on a more "fibroblast-like" shape. Growth was very vigorous with numerous mitoses and, as the cell numbers increased, they piled up on one another in many layered aggregates. The cell line was in its ninth passage, five months after initiation, when incubator failure terminated the cultures.

Fig. 199.
CTH 1. Pass 1. Day 1.
Phase contrast. Original magnification x 150

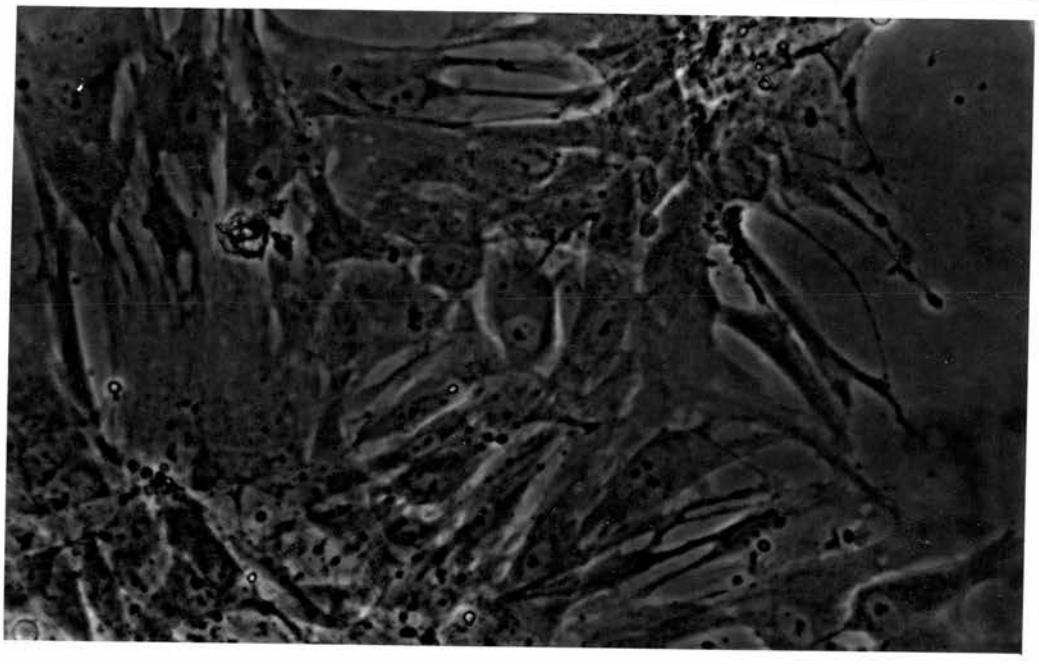


Fig. 200.

CTH 1. Pass 1. Day 6, showing numerous mitoses.

Phase contrast. Original magnification x 150

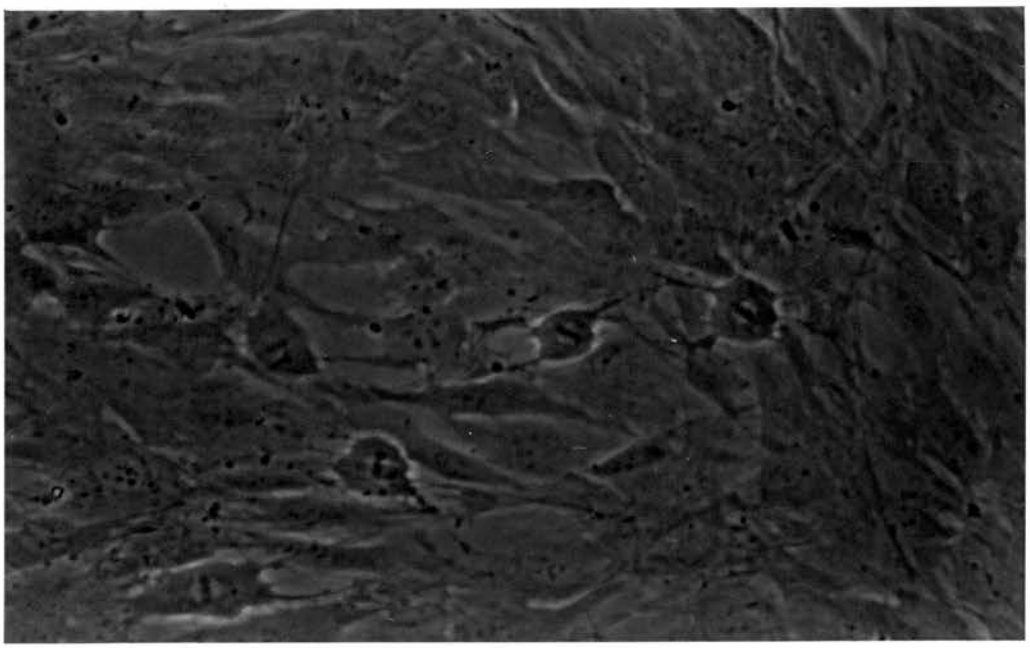
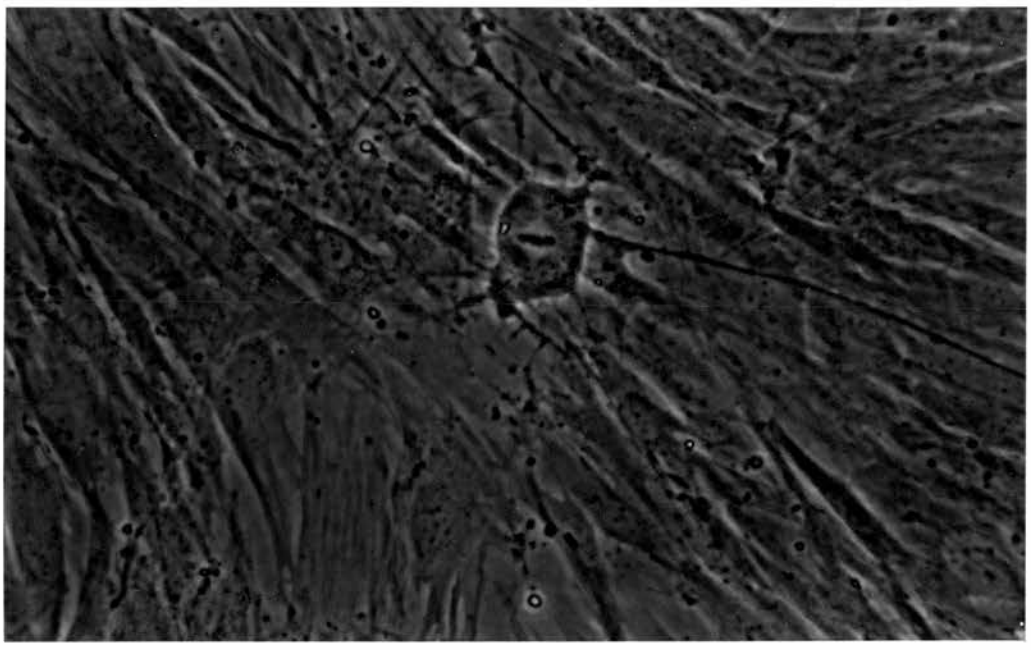


Fig. 201.

CTH 1. Pass 1. Day 6. Large mitotic figures.

(Same magnification as above)

Phase contrast. Original magnification x 150

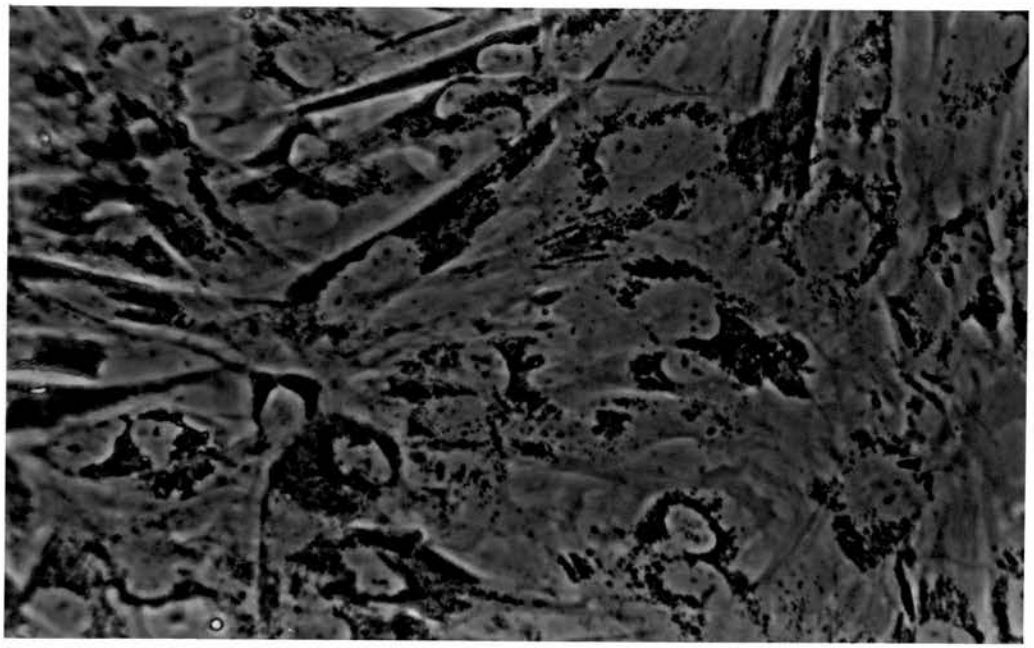


When the cells of CTH 1 were exposed to the vital dye - Neutral Red, they took the stain up into the cytoplasm in a perinuclear position.

Fig. 202.

CTH 1. Pass 1. Day 9. Uptake of Neutral Red.

Phase contrast. Original magnification x 150



When the cells of CTH 1 were trypsinised they were often released from the flask floor in clumps and appeared to be held together by something. When the cell layers were stained for the presence of reticulin, using Gordon and Sweets' silver impregnation method, networks of reticulin were demonstrated above the cell layers. The capacity to produce reticulin fibrils was retained throughout the entire culture period.

Further photographic evidence of reticulin production by CTH 1 cells is included in Section II (p 116).

Fig. 203.

CTH 1. Pass 6. Day 14. Phase contrast of stained preparation.

Original magnification x 150

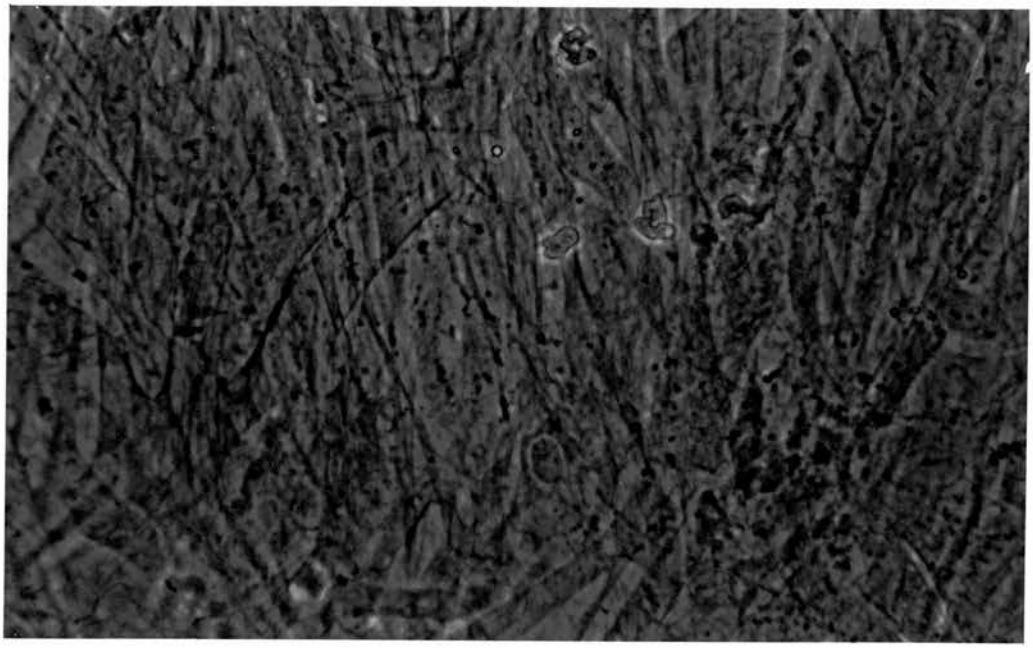
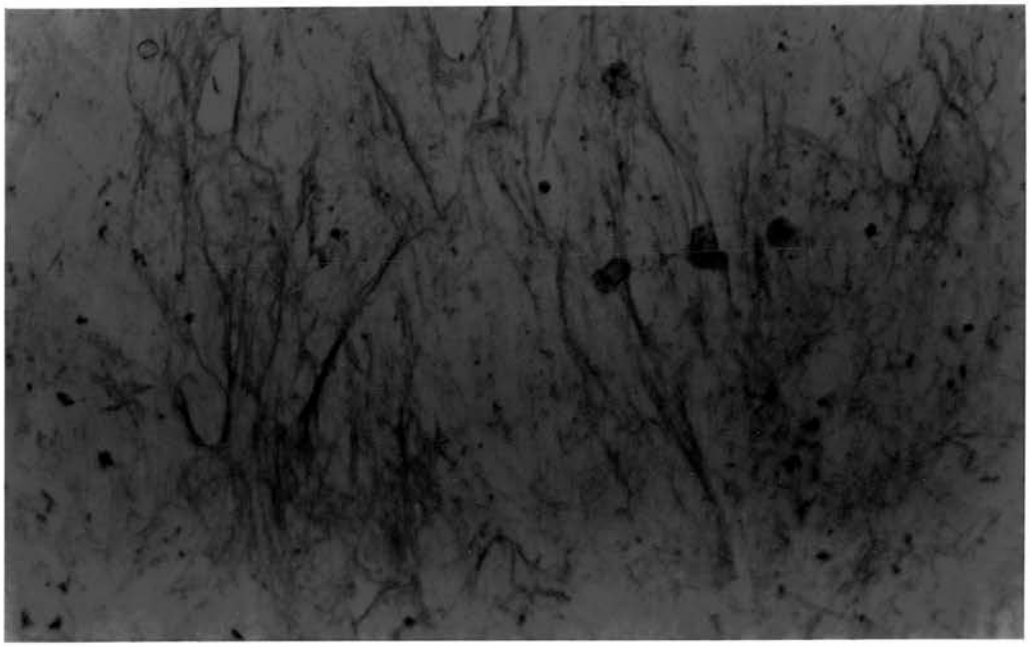


Fig. 204.

As above, viewed with plain light, showing reticulin fibres stained with silver.

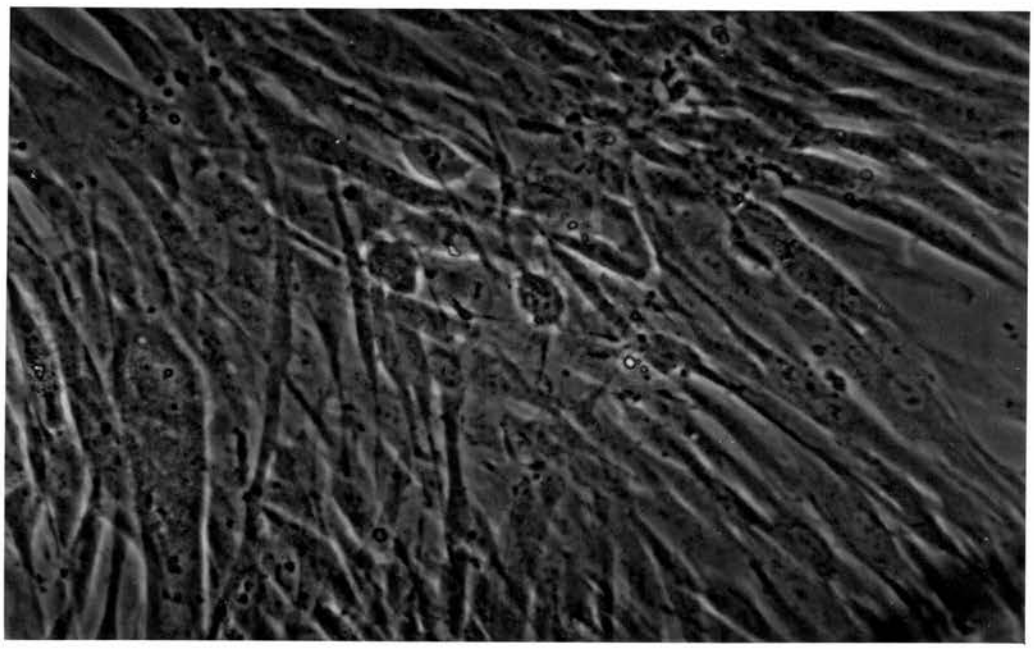
Original magnification x 150



CTH 7.

CTH 7 was derived from an anaplastic carcinoma (TC 57), 38 days after initiation of the original culture. The cell line grew vigorously through seven passages over a period of 3 months before incubator failure destroyed it. The cells of CTH 7 were fibroblast-like in appearance but, unlike classical fibroblasts, at high cellular concentration, they repeatedly contracted into ridges and lumps. CTH 7 cells were most probably myofibroblasts as described by Majno[106].

Fig. 205.
CTH 7. Pass 4. 7 days.
Phase contrast. Original magnification x 150



Lumps of CTH 7 cells were taken at various passages, fixed in formalin and processed histologically (see p. 34). The application of various stains revealed the presence within the cell lumps of a variety of connective tissue proteins.

Fig. 206. CTH 7 cells. 7 days culture.
Phase contrast. Original magnification x 150

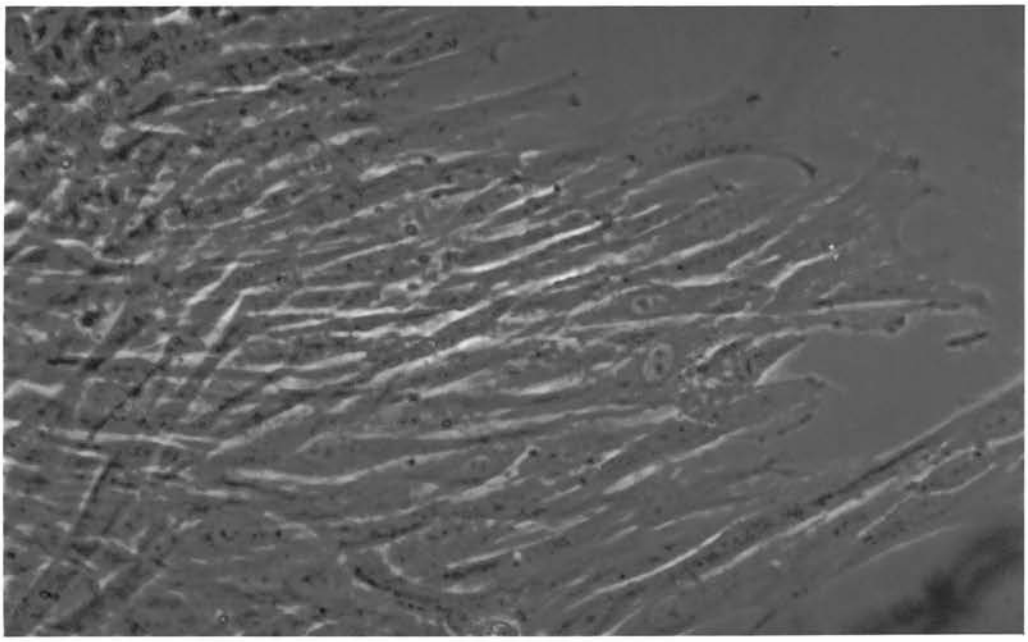


Fig. 207.

H & E Original magnification X 14.4

Histological section of a cellular aggregate of CTH 7 cells.

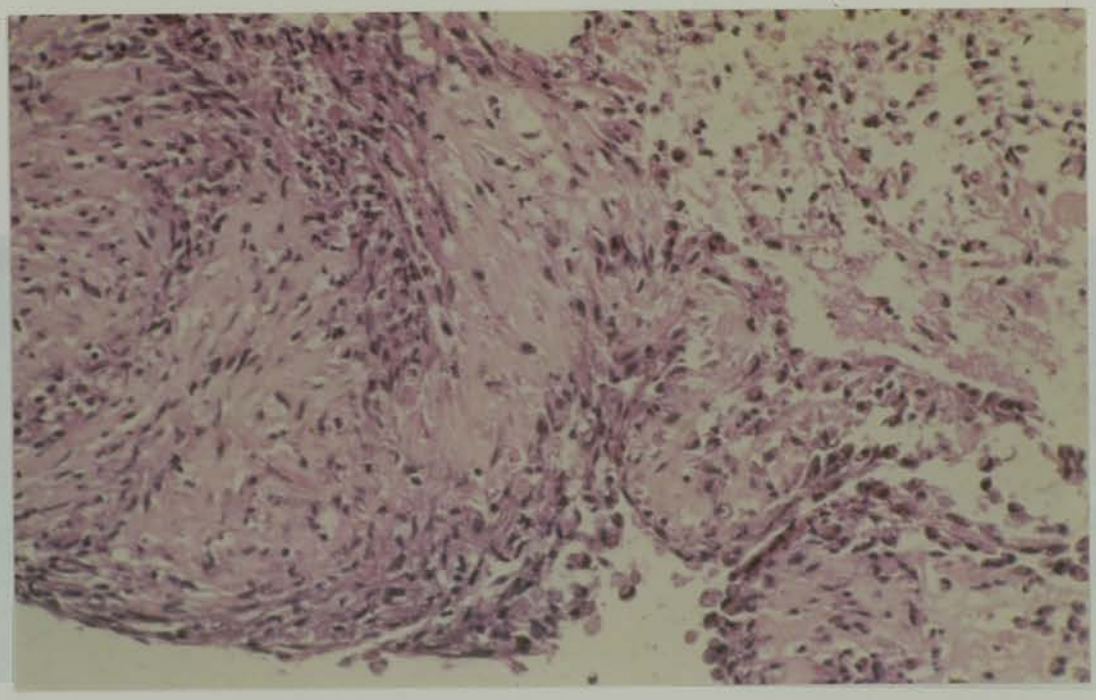


Fig. 208.

Histological section of a cellular aggregate of CTH 7 cells.

H & E Original magnification X 72

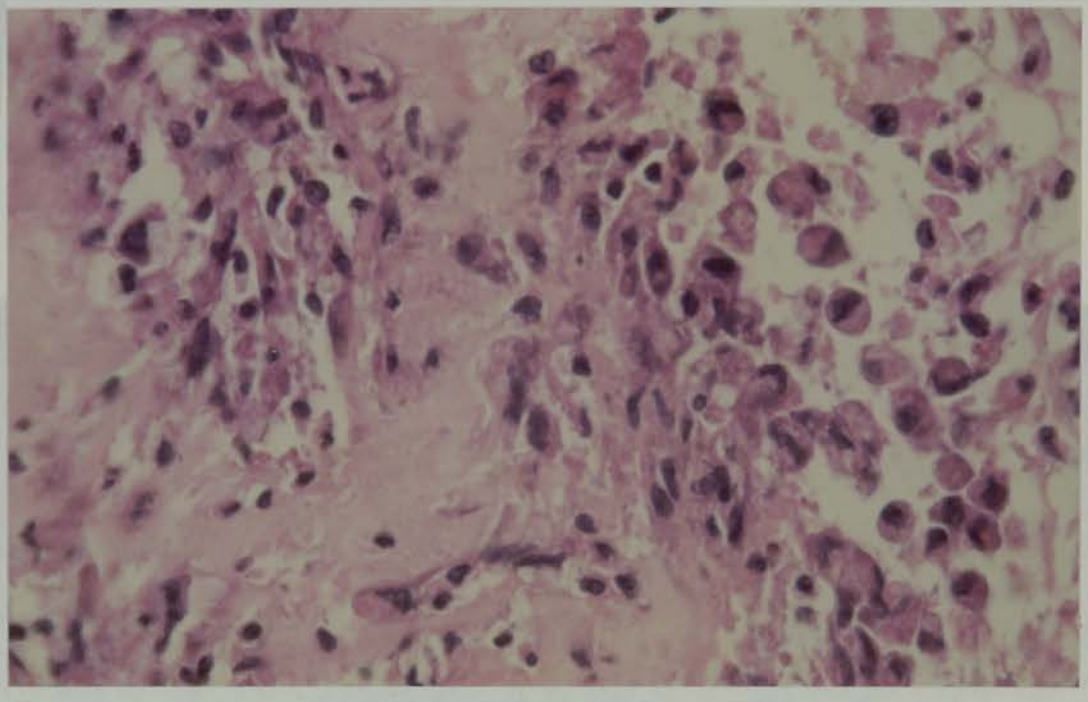


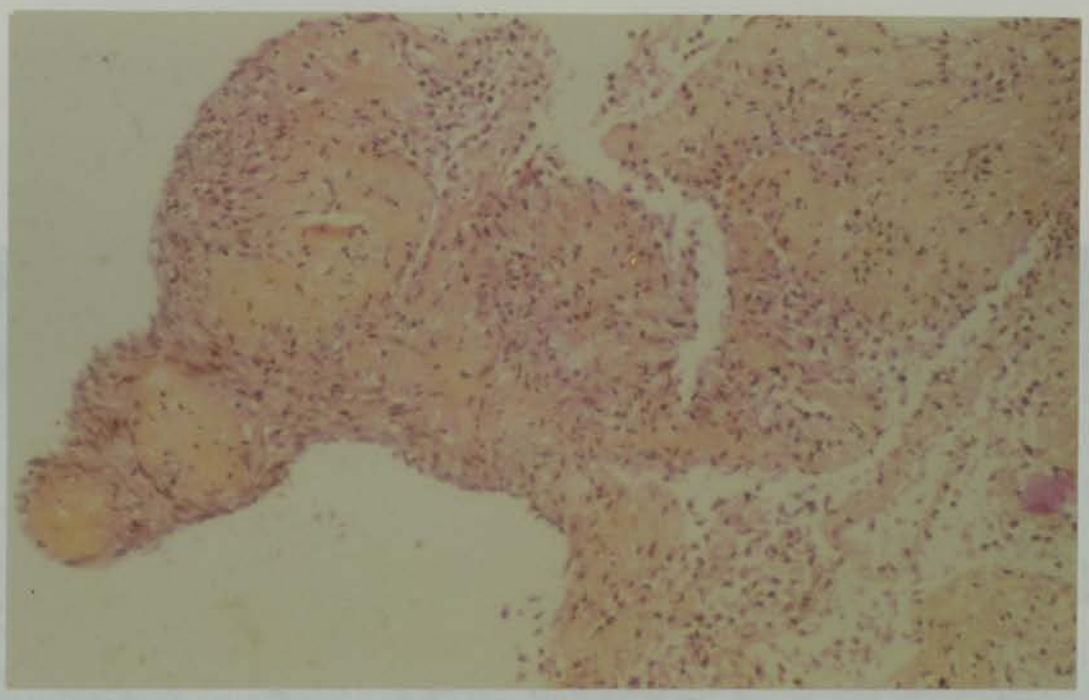
Fig. 209.

Histological section of a cellular aggregate of CTH 7 cells stained to show the presence of collagen.

Fig. 210.

Histological section of a cellular aggregate of CTH 7 cells stained to show the presence of collagen.

Haematoxylin, phloxine and saffron. Original magnification X 14.4



Haematoxylin, phloxine and saffron. Original magnification X 72

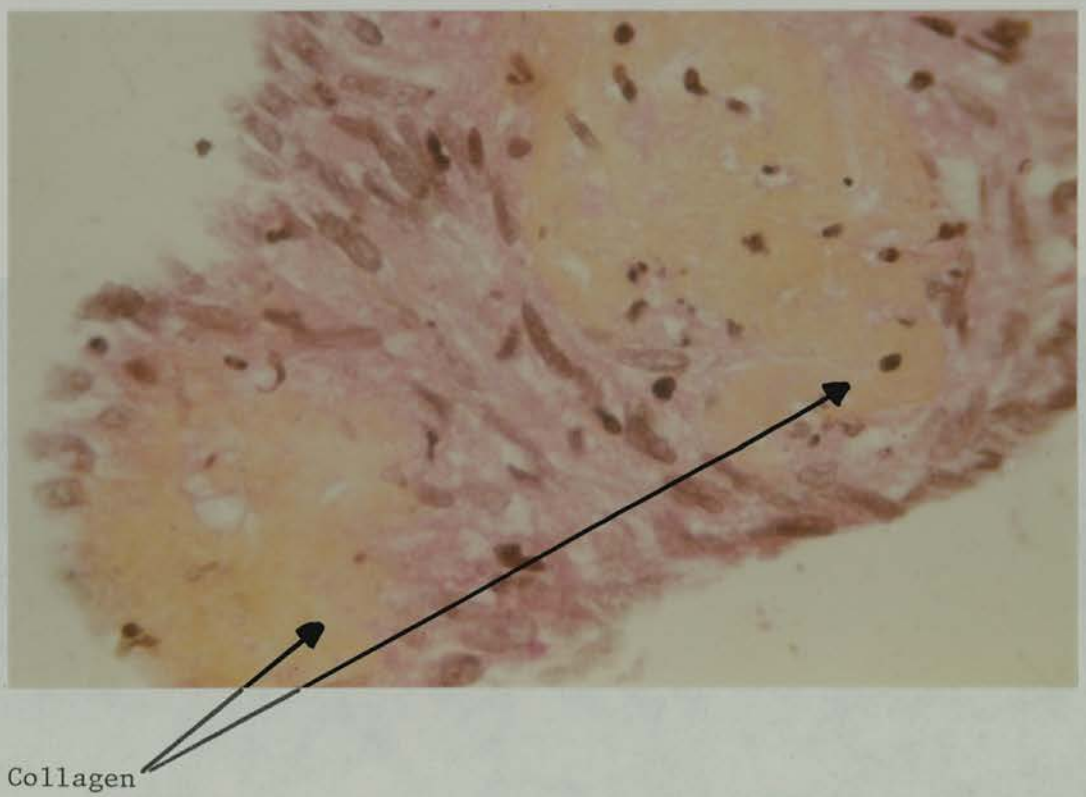


Fig. 211.

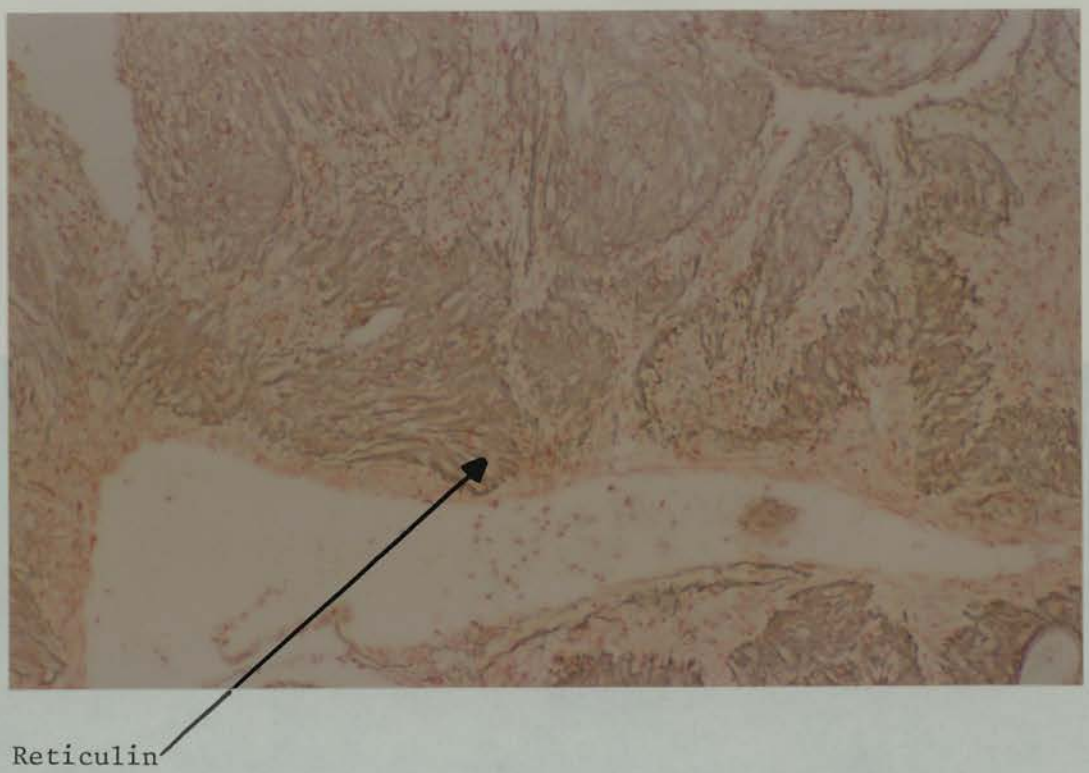
Histological section of a cellular aggregate of CTH 7 cells stained to show the presence of reticulin.

Fig. 212.

Histological section of a cellular aggregate of CTH 7 cells stained to show the presence of elastin.

Gordon and Sweets' silver impregnation for reticulin/neutral red.

Original magnification X 14.4



Gomori's aldehyde fuchsin for elastin/tartrazine.

Original magnification X 14.4

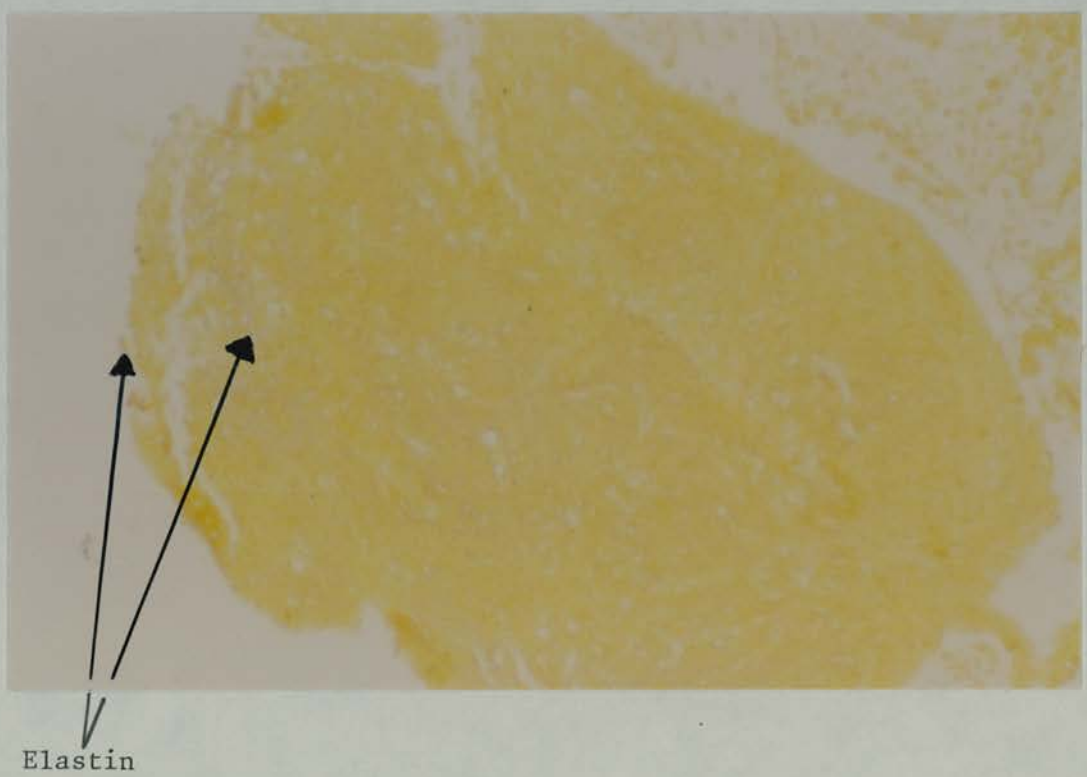


Fig. 213.

CTH 7 cells. Gordon and Sweets' silver impregnation method for the demonstration of reticulin fibres. Phase contrast.

Original magnification x 150

In addition, as recorded in Section II, fibrillar reticulin could be demonstrated above the CTH 7 cells throughout all cell passages. Phase contrast of stained preparation.

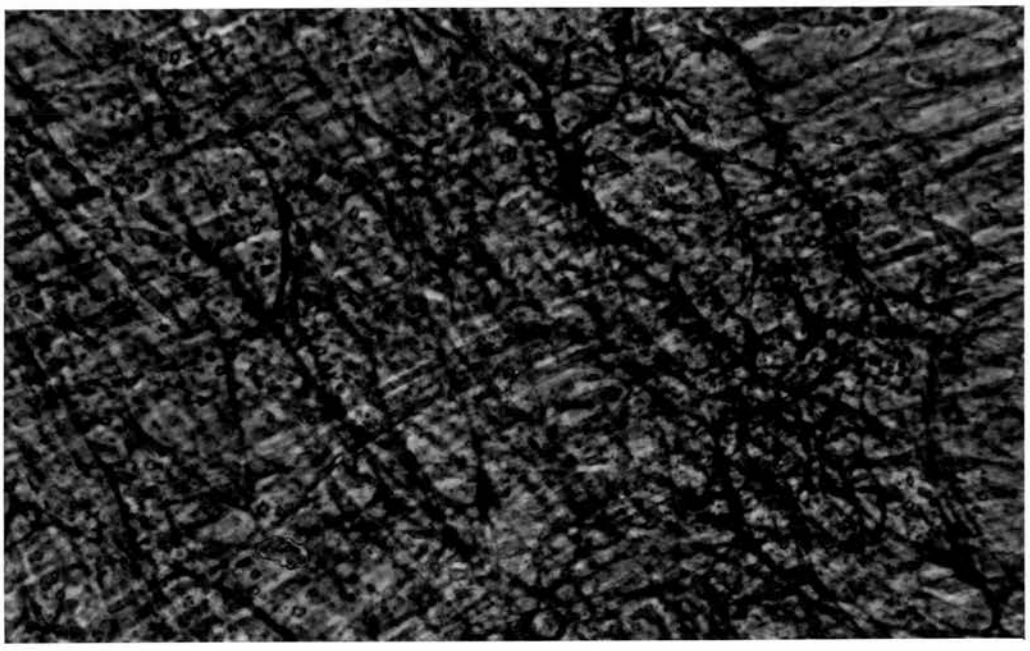


Fig. 214.

As above, viewed with plain light, showing fibrils of reticulin stained with silver.

Original magnification x 150

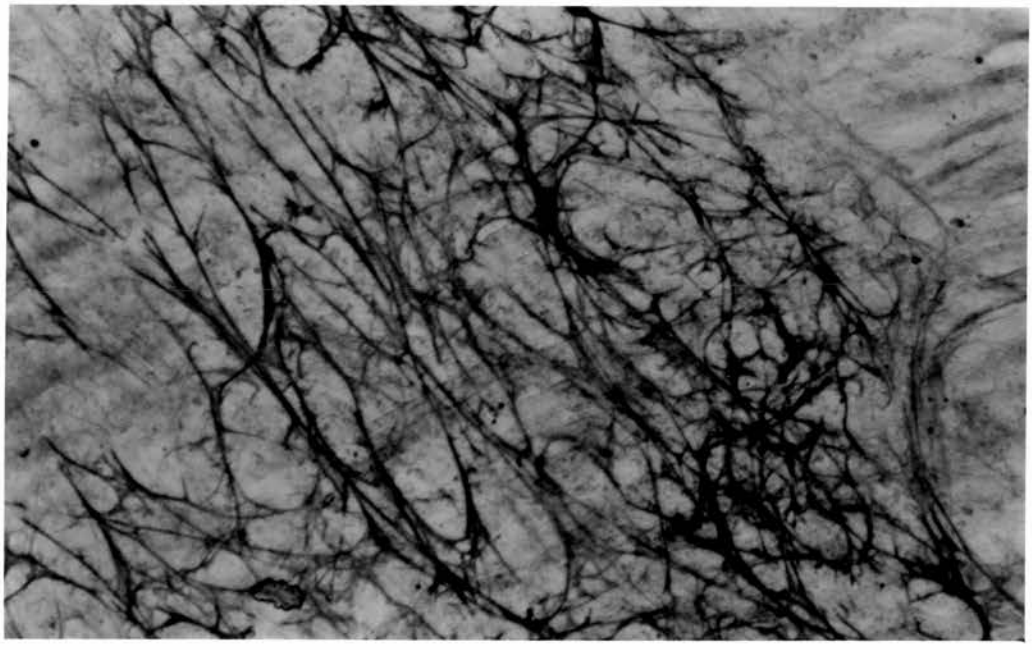


Fig. 215.

CTH 7. Network of reticulin fibres extending across the cell sheet.

Plain light. Original magnification x 150

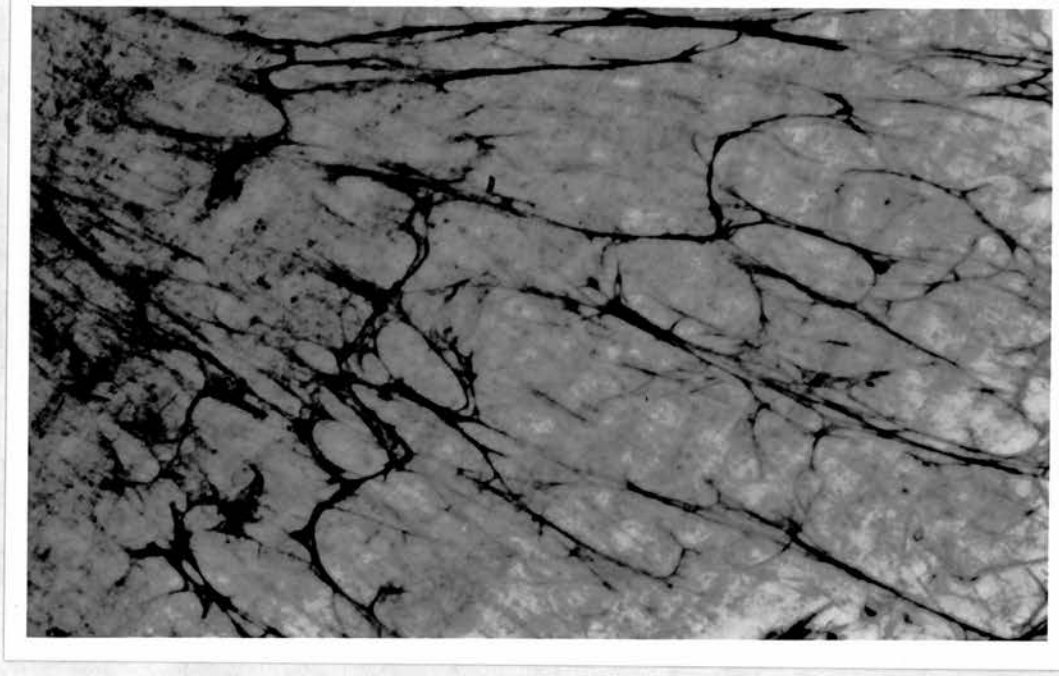


Fig. 216.

Phase contrast of the same area.

Original magnification x 150

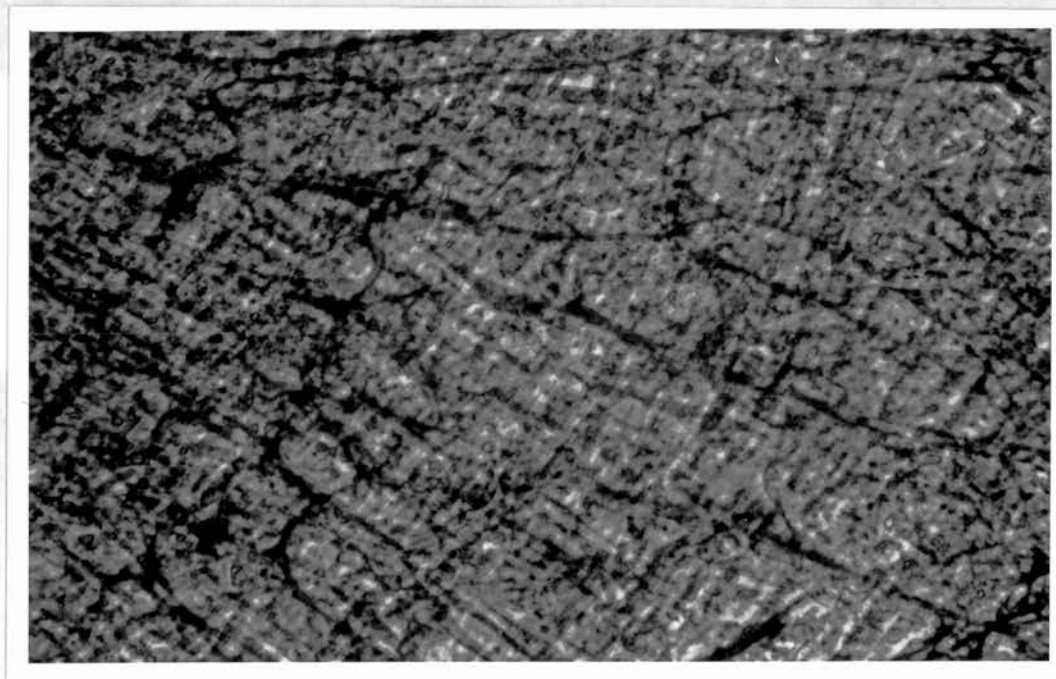


Fig. 217.

'Typical epithelial group'. Phase contrast. Original magnification x 150

SECTION VI

THE MORPHOLOGY AND BEHAVIOUR OF THE CELL TYPES GROWN

The cells which grew from the biopsy and mastectomy specimens did not divide neatly into epithelial cells and fibroblasts but covered a wide range of morphological types. The cells could be loosely assigned to 16 groups according to their appearance and behaviour. Most cell cultures contained more than one cell type. The distribution of each cell type is given in Tables II, III and IV.

For ease of identification simple descriptive titles have been given to the cell types. No specific biological significance should be inferred from these terms.

TYPE I. "Typical Epithelial Groups".

Typical epithelial groups were most often seen in the first few days of growth and consisted of small groups of fairly regular polygonal cells closely abutted one to another. The nuclei were oval, pale in colour and contained one or two prominent, dark nucleoli. This type of cell often developed into either the "elongated epithelial" or the "bridged epithelial" cell type and was probably a transient early cultural cell type which appeared before the true cultural morphology had developed.

TC 33. Anaplastic, small cell carcinoma, moderate "stromal reaction", moderate elastosis. 1 day's culture.

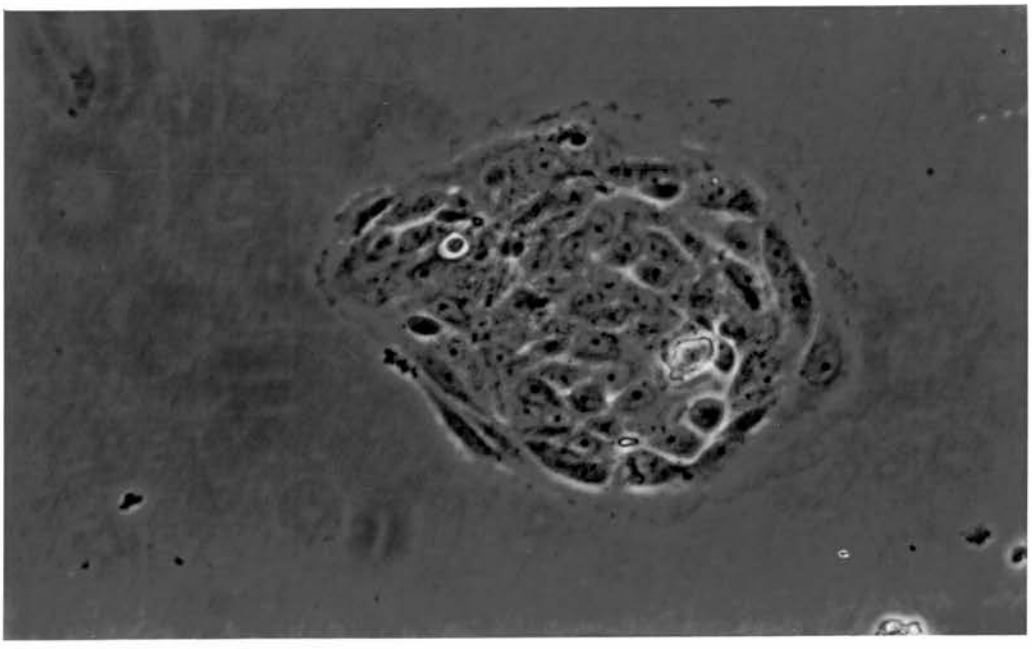


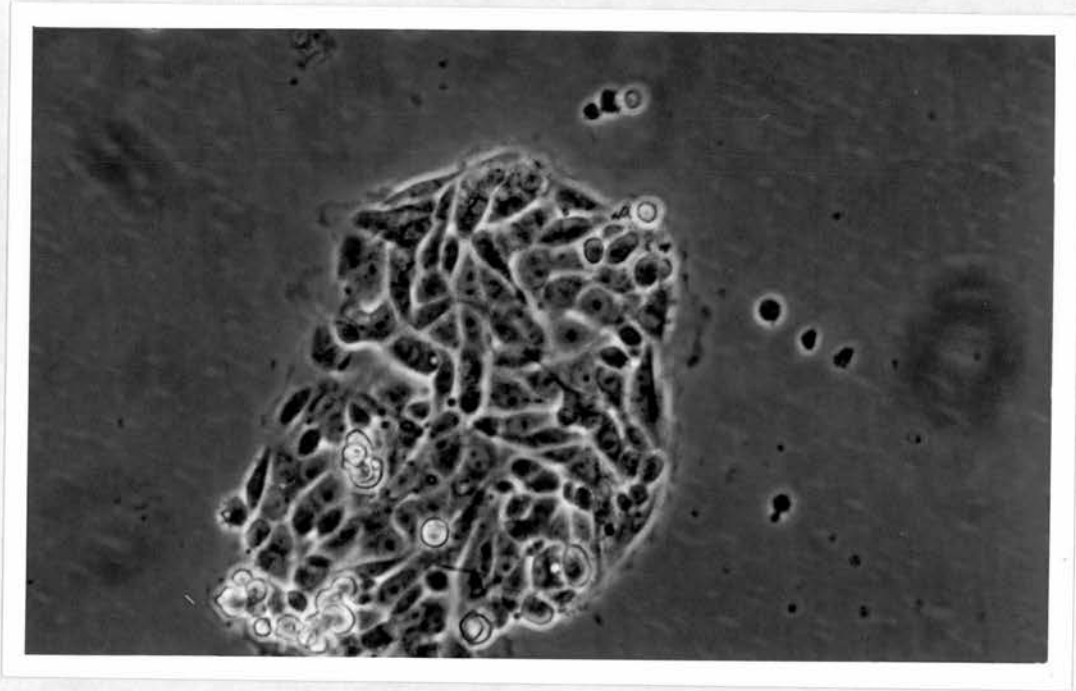
Fig. 218.

'Typical epithelial group'. Phase contrast. Original magnification x 150

Fig. 219.

'Typical epithelial group'. Phase contrast. Original magnification x 150

TC 70. Gynaecomastia. 1 day's culture.



TC 19. Fibroadenosis. 1 day's culture.

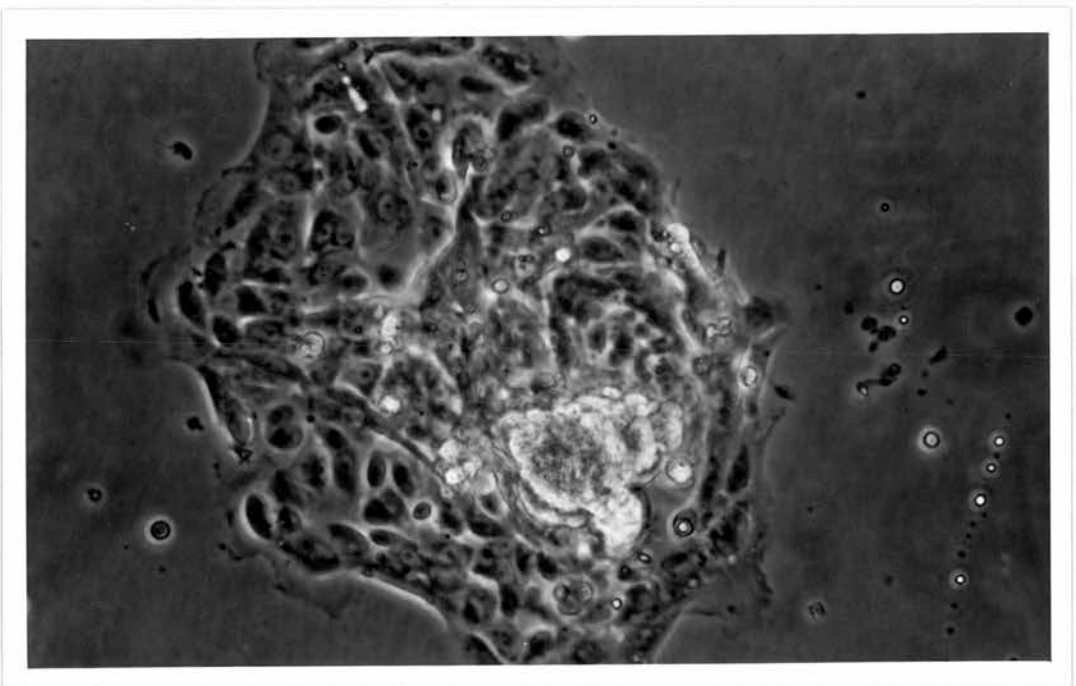


Fig. 220.

Elongated epithelial cells. Phase contrast. Original magnification x 150

TYPE II. "Elongated Epithelial Cells".

Elongated epithelial cells were similar to the typical epithelial cells but presented an overall appearance of long, thin cells as opposed to typically polygonal cells, with length:breadth ratios of 1:3 to 1:5. Once again, the nucleus of these cells was oval, pale and contained one or two prominent, dark nucleoli. This type of cell often showed a marked intercellular refringence - a feature which has been shown to be indicative of the presence of connective tissue (see Section II, p 116). The growth of this type of cell tended to be limited and eventually the cells became "bound" by reticulin and growth ceased. Elongated epithelial cells were found in cultures of both benign and malignant tissue.

Cells similar to those illustrated were designated 'E' cells by Hallowes et al [73] and were cultured by them from lacteal secretions, benign mammary tissues and also primary carcinomas. Hallowes also found that the growth of 'E' cells was limited and ceased, as judged by the size of the cell islands, after the third week in culture.

TC 9. Fibrocystic disease. 7 days culture.

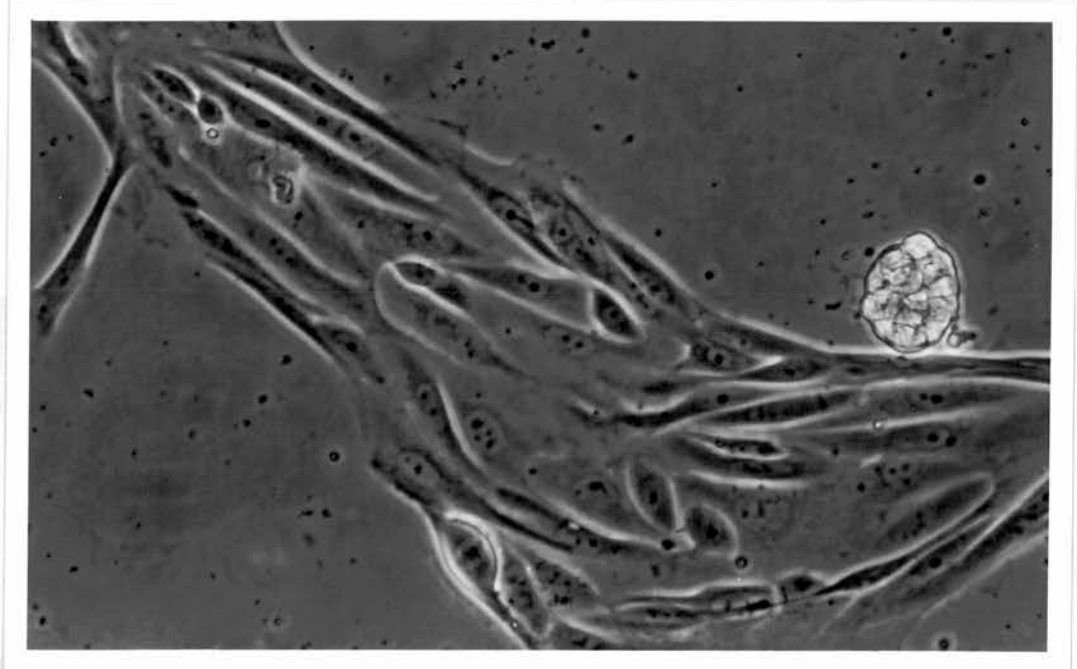


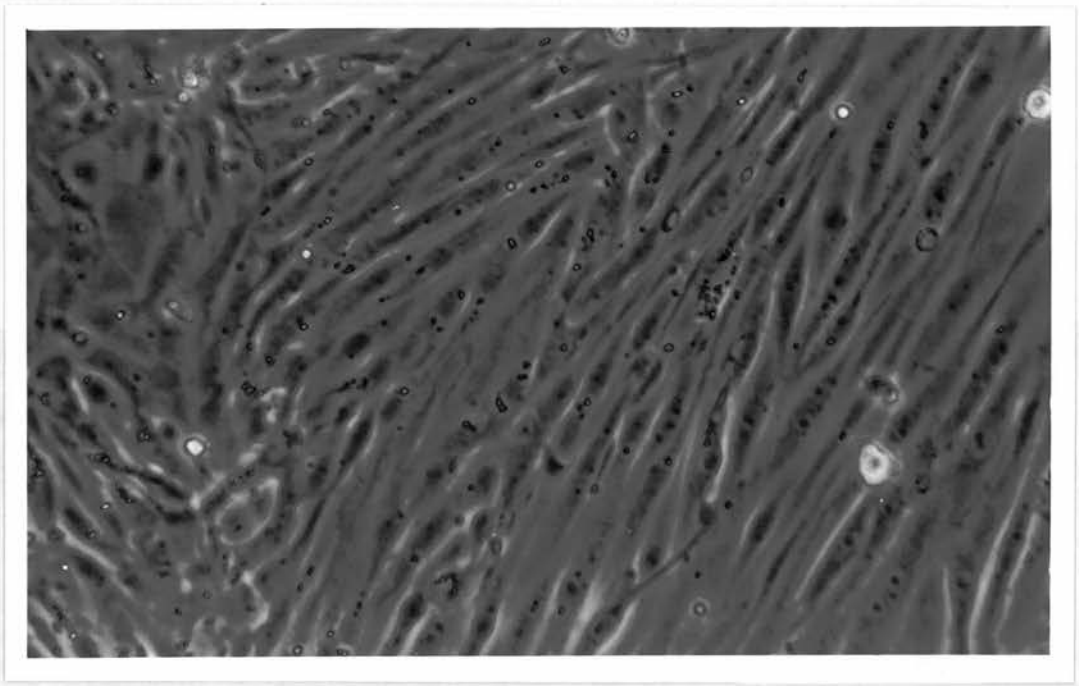
Fig. 221.

Elongated epithelial cells. Phase contrast. Original magnification x 150

Fig. 222.

Elongated epithelial cells. Phase contrast. Original magnification x 150

TC 11. Fibroadenosis. 6 days culture.



TC 21. Anaplastic, medullary carcinoma. 5 days culture.

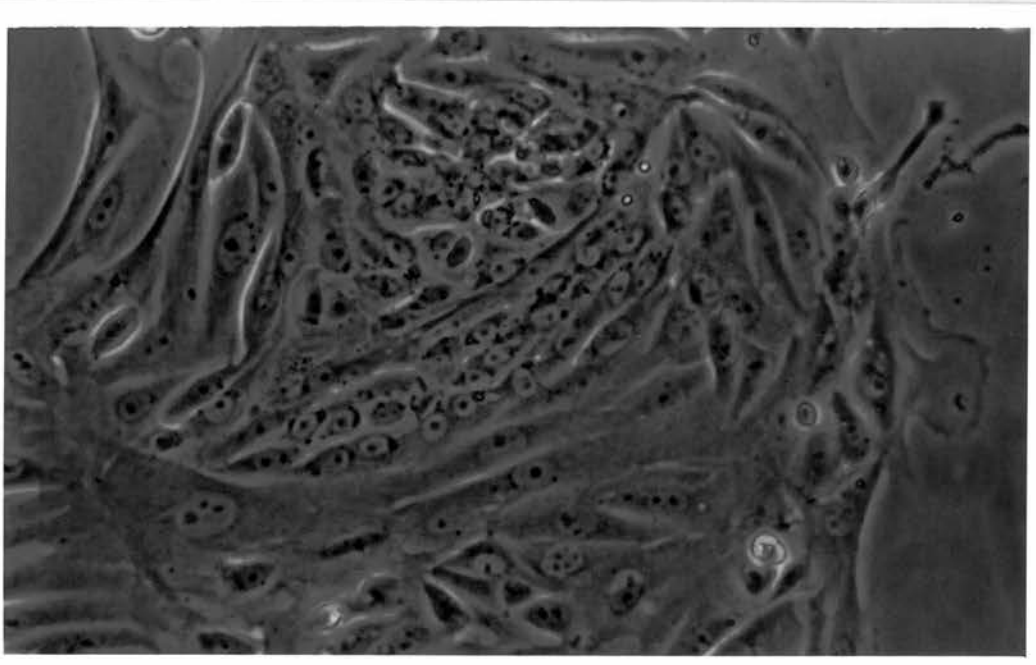


Fig. 223.

Elongated epithelial cells. Phase contrast. Original magnification x 150

TC 41. Anaplastic carcinoma. Mild/moderate "stromal reaction".
6 days culture. Formalin fixed preparation.

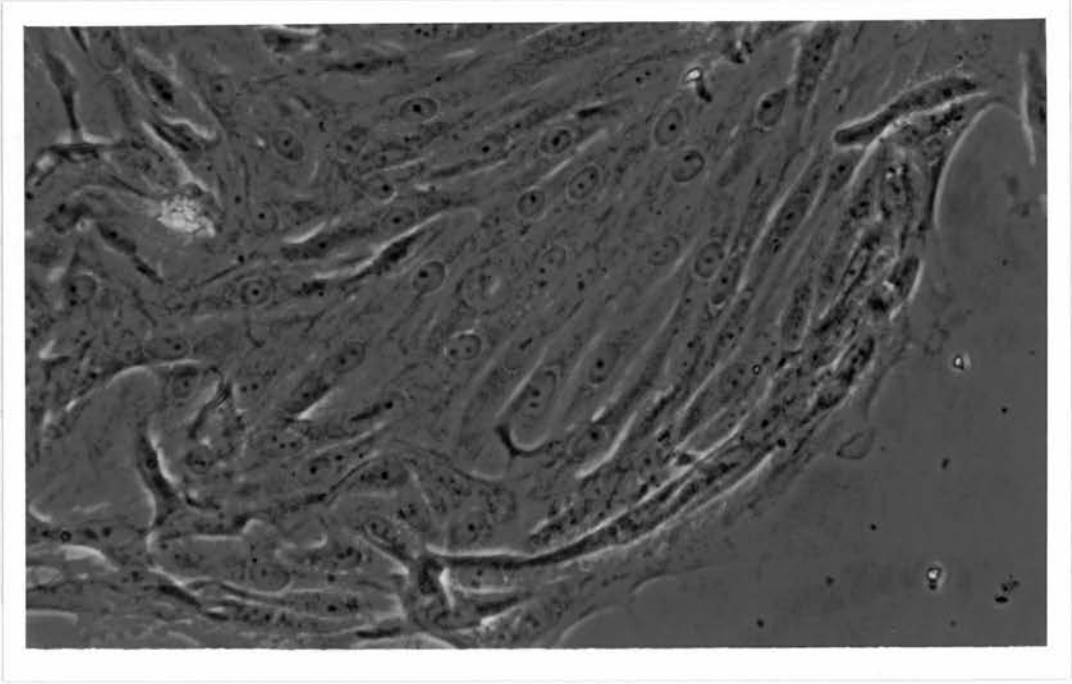


Fig. 224.

'Bridged epithelial cells'. Phase contrast. Original magnification x 150

TYPE III. "Bridged Epithelial Cells"

Bridged epithelial groups consisted of a mixture of polygonal and elongated cells in a pavement within which existed areas where an individual cell projected a long extension to another part of the cell pavement, thus forming a cellular "bridge". In some instances the cellular bridges extended for several microns across the floor of the culture vessel, thus joining groups of cells together. At later incubation periods the bridges often exhibited refringence. Bridged epithelial cell pavements occurred in cultures of both benign and malignant tissues.

TC 19. Fibroadenosis. 6 days culture.

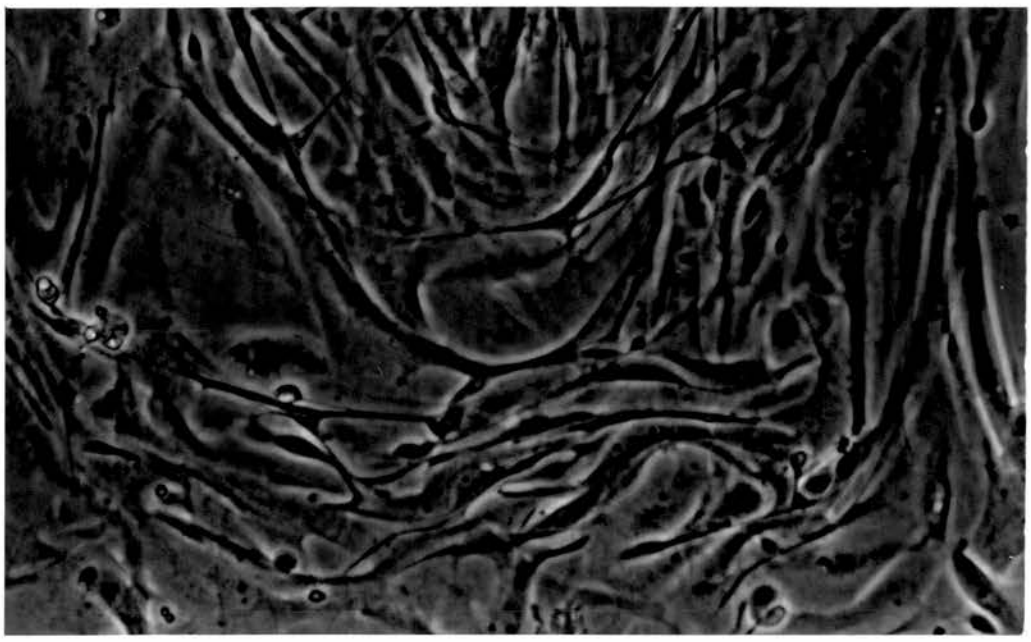


Fig. 225.

'Bridged epithelial cells'. Phase contrast. Original magnification x 150

TC 79. Anaplastic carcinoma. Large cell type. Slight to moderate "stromal reaction". 4 days culture.

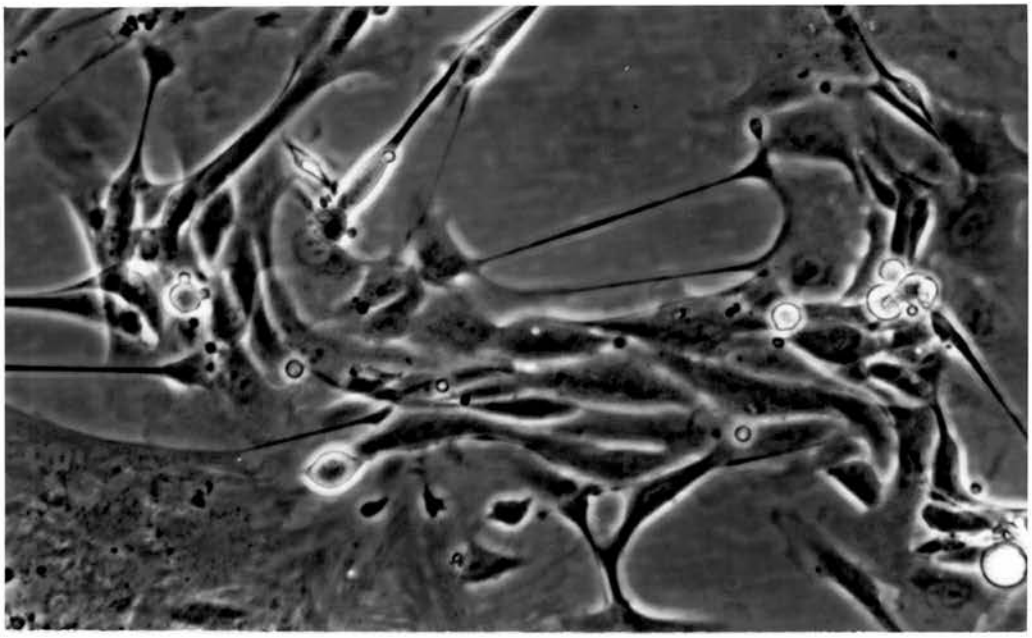


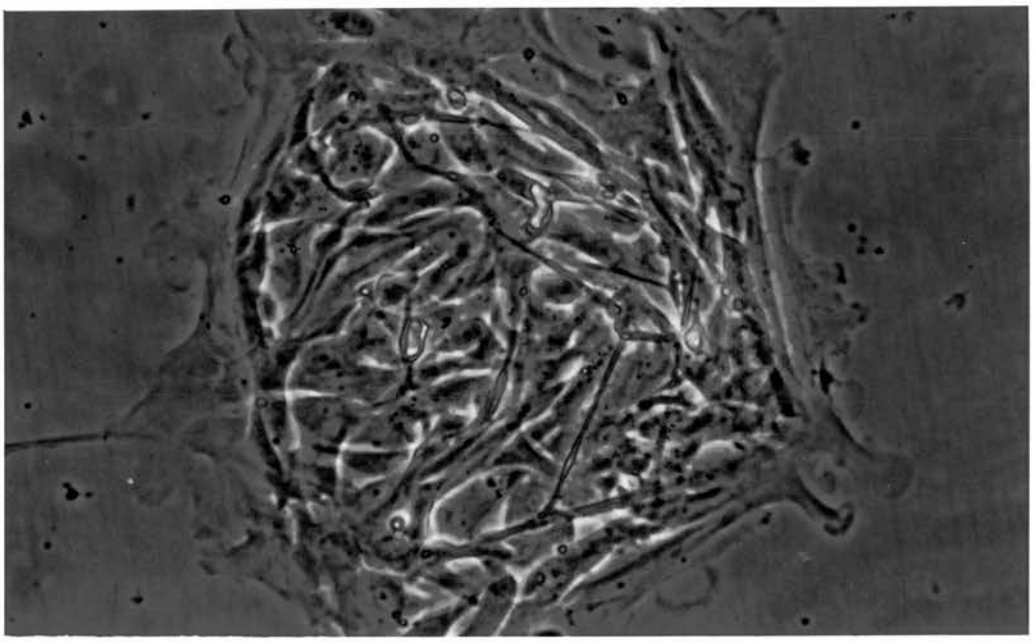
Fig. 226.

'Bridged epithelial cells'. Phase contrast. Original magnification x 150

Fig. 227.

'Bridged epithelial cells'. Phase contrast. Original magnification x 150

TC 33. Anaplastic, small cell carcinoma. Moderate "stromal reaction", moderate elastosis. 7 days culture.



TC 75. Anaplastic carcinoma. Little "stromal reaction". 14 days culture.

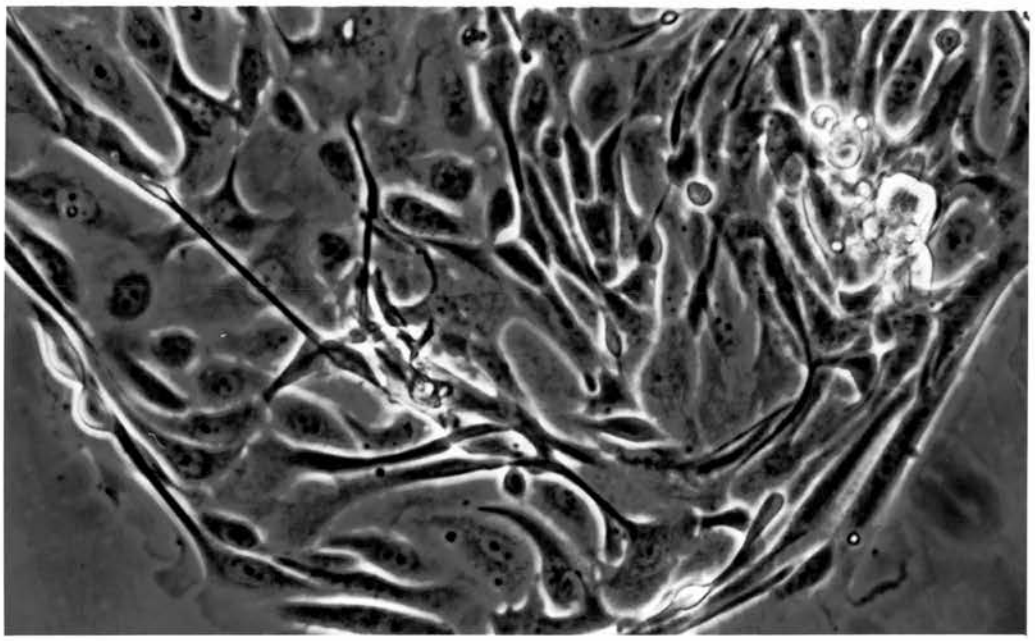


Fig. 228.

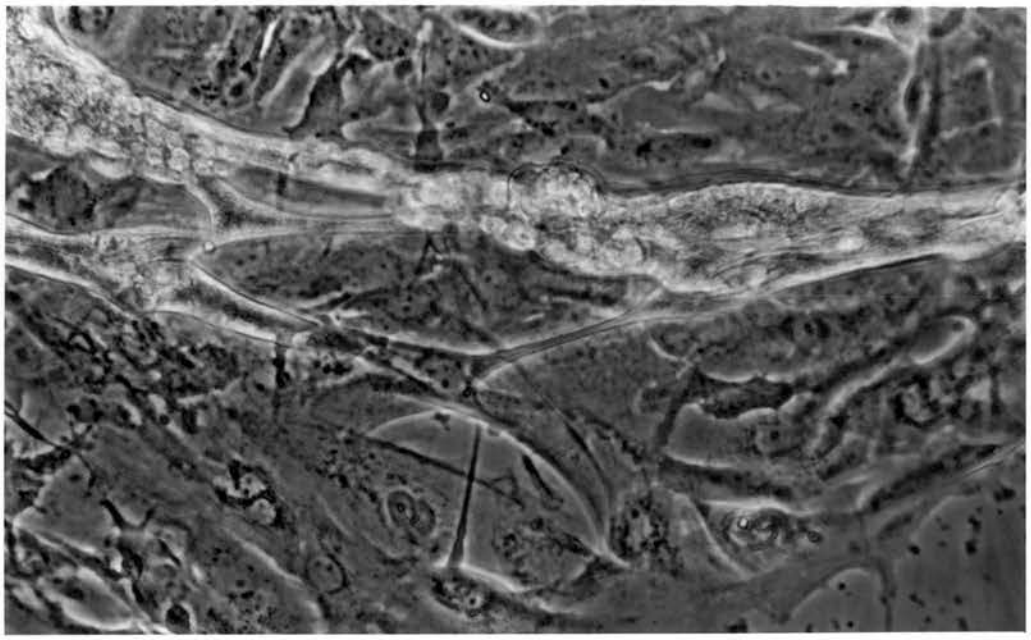
'Bridged epithelial cells'. Phase contrast. Original magnification x 150

Fig. 229.

Detailed view of an intercellular bridge.

TC 23. Anaplastic carcinoma, partly intraduct.

29 days culture.



When viewed with the scanning electron microscope, the surface of the bridges was shown, in one case at least, to be covered with minute nodules, approximately 0.01 µm in diameter.

TC 82. Highly elastotic anaplastic carcinoma. 20 days culture.

Detail of an intercellular bridge.

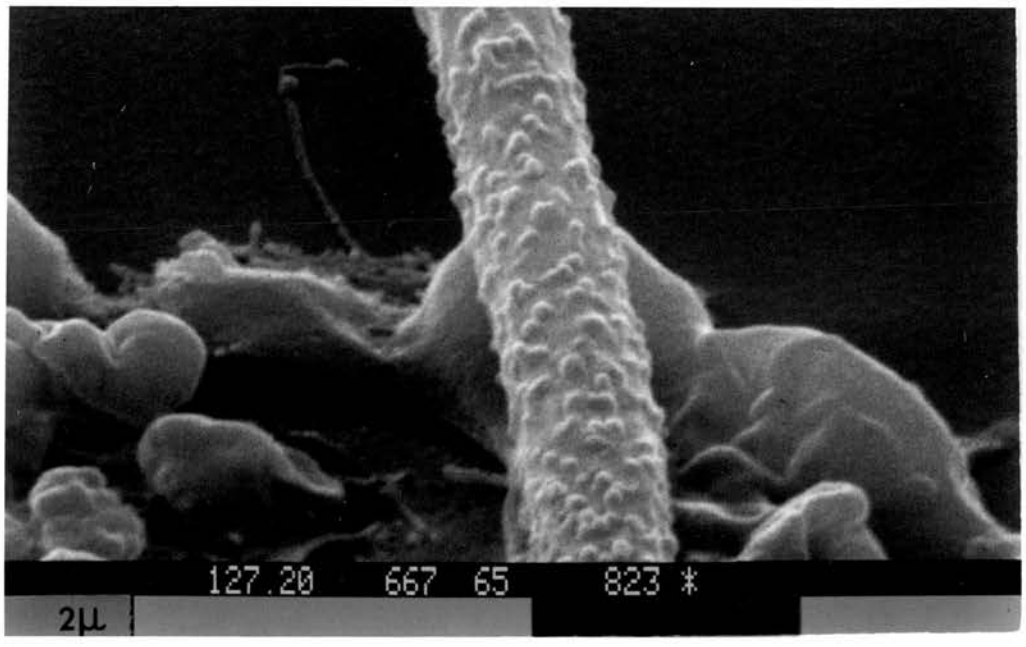


Fig. 230.

Neat epithelial cells. Phase contrast. Original magnification x 150

TYPE IV. "Neat Epithelial Cells"

Cells of this type were remarkable for the regularity of their appearance. They had large, pale oval nuclei with one or two very prominent dark nucleoli and a high nuclear:cytoplasmic ratio. There was often a tendency towards granularity in the perinuclear area and it may be that this type of cell and the "Granular" cells (Type V. p 202) are the same cell but in different stages of development. Neat epithelial cells were found in cultures of both benign and malignant tissues.

TC 33. Anaplastic, small cell carcinoma. Moderate "stromal reaction", moderate elastosis. 9 days culture.

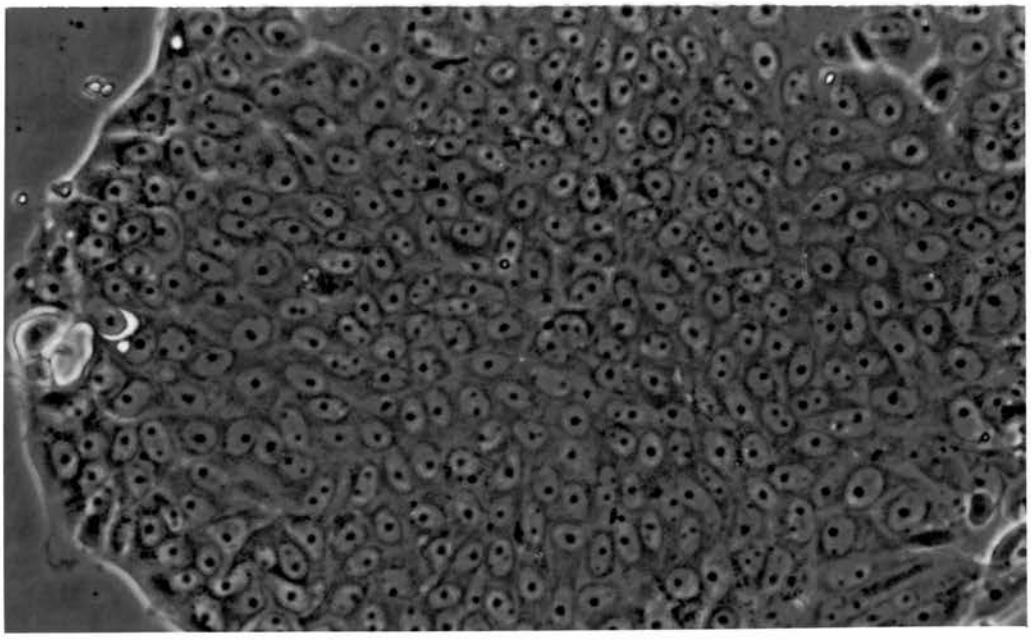


Fig. 231.

Neat epithelial cells. Phase contrast. Original magnification x 150

TC 39. Scirrhous carcinoma. 3 days culture.

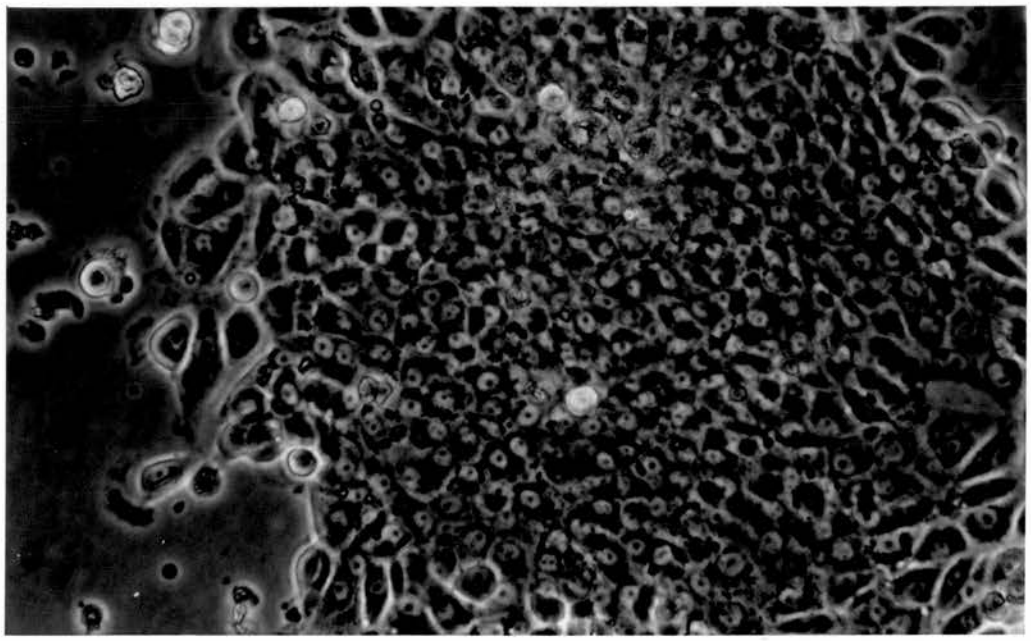


Fig. 232.

Granular epithelial cells. Phase contrast. Original magnification x 150

TYPE V. "Granular Epithelial Cells"

Granular cells were epithelial cells of a regular appearance which had a pale, almost circular nucleus containing one or more dark nucleoli. The diagnostic feature of these cells was the presence of a zone of very granular cytoplasm surrounding and immediately adjacent to the nucleus. Granular cells occurred in all cultures from tissue containing an apocrine component and are probably synonymous with apocrine type cells. A full description of the growth and behaviour of this type of cell is contained in Section III (p 143).

TC 76. Highly elastotic anaplastic carcinoma with an apocrine component. 30 days culture.

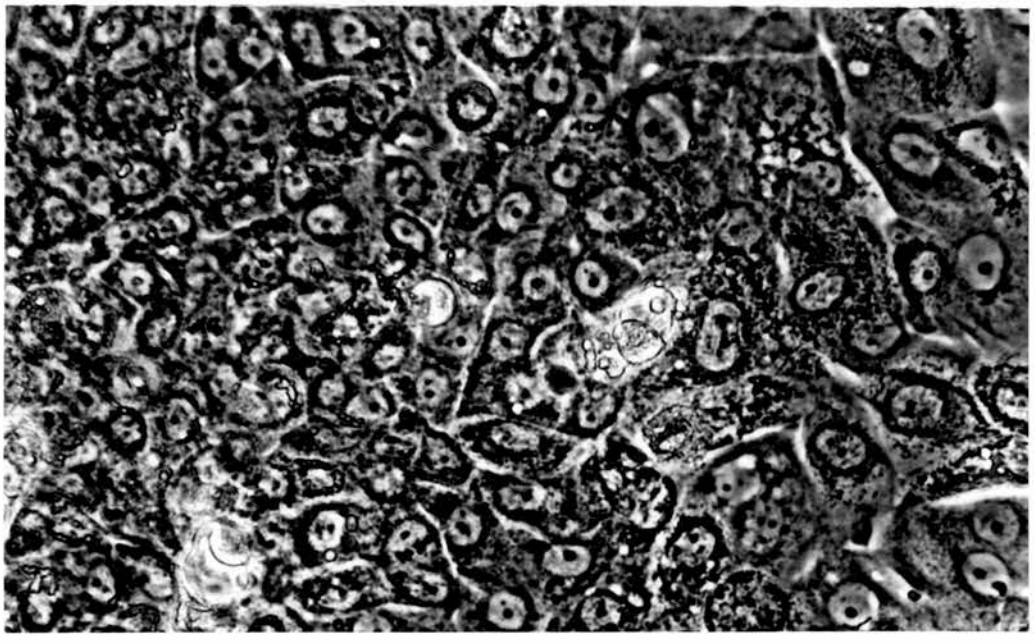


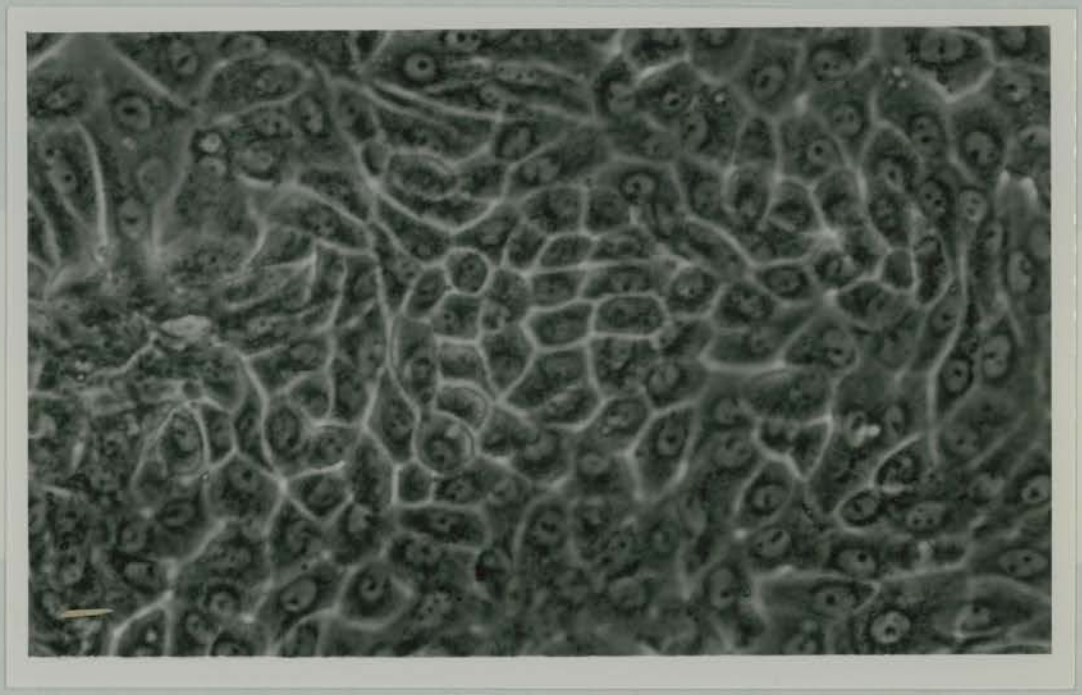
Fig. 233.

Granular epithelial cells. Phase contrast. Original magnification x 150

Fig. 234.

Granular epithelial cells of TC 51, an apocrine variant of mammary carcinoma, stained with Oil Red O to demonstrate the presence of lipid. Plain light. Original magnification x 150

TC 51. Apocrine variant of mammary carcinoma. 9 days culture.



Staining with Oil Red O (pp 40) revealed small accumulations of fat in some of the granular cells.

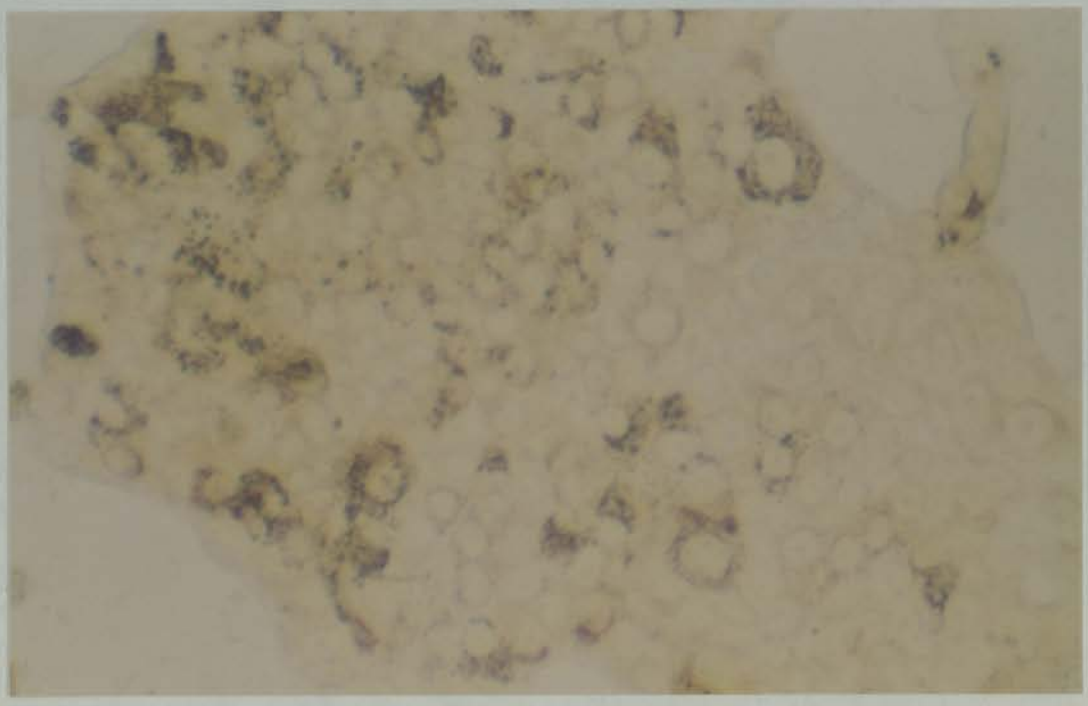


Fig. 235.

Very pleomorphic cell pavement. Phase contrast. Original magnification x 150

TYPE VI. "Very Pleomorphic Cell Pavements".

This type of cell pavement contained cells of all morphological types. There was no well defined cell which could be looked upon as dominant. The cells often piled up on one another in multiple layers, mitoses were frequent and often very bizarre. Although this type of cell pavement did occur in cultures from non-neoplastic tissue they were more common in those cultures originating from cancerous tissue.

TC 1. Lobular, intraduct carcinoma. 9 days culture.

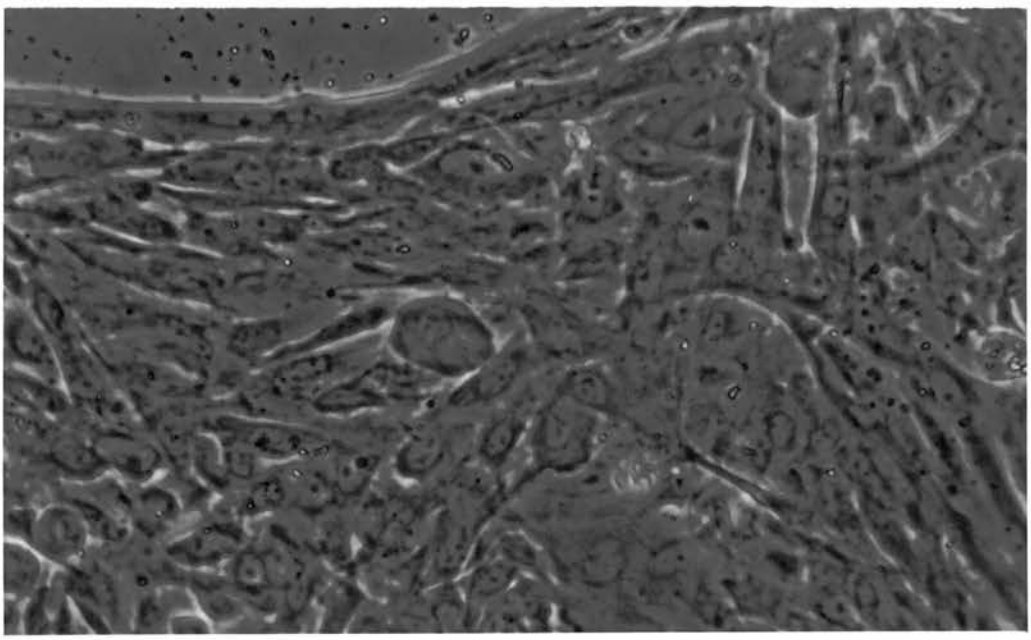
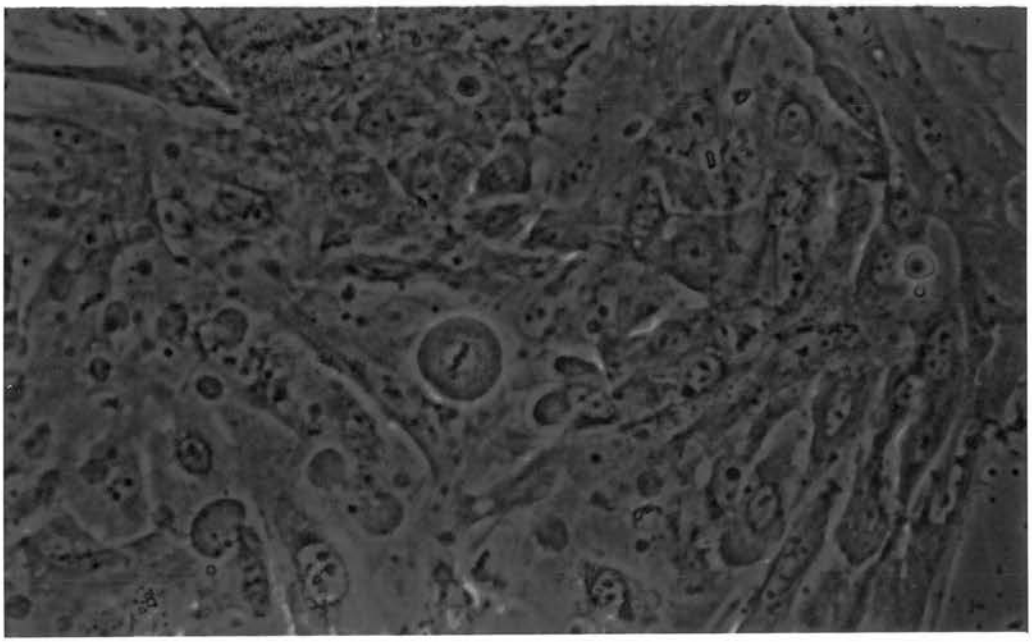


Fig. 236.
As above. 20 days culture, showing a cell in mitosis.



Phase contrast. Original magnification x 150

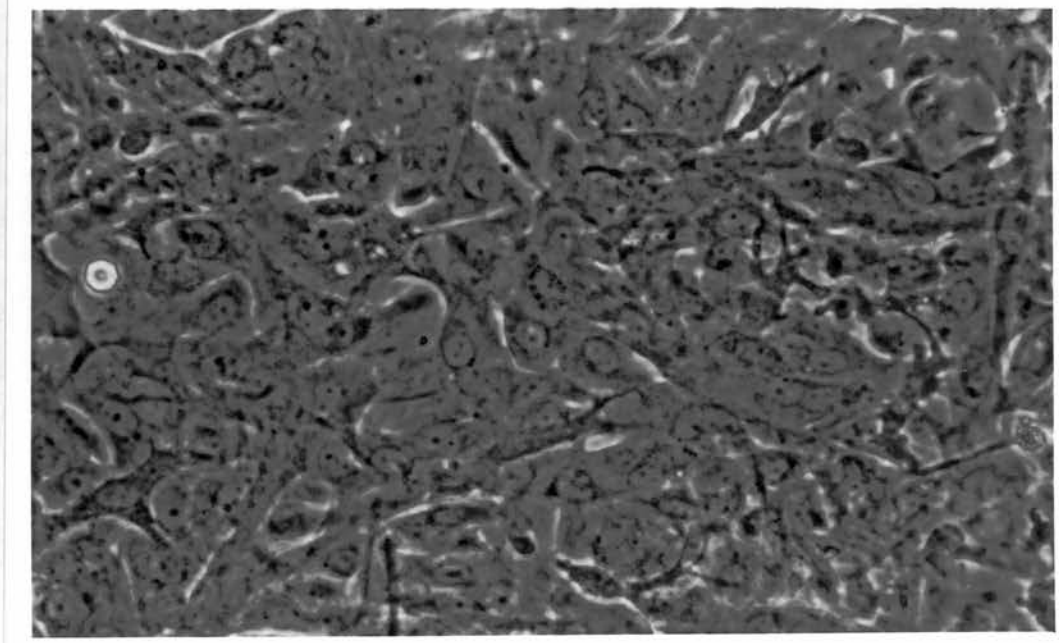
Fig. 237.

Very pleomorphic cell pavement. Phase contrast. Original magnification x 150

Fig. 238.

Very pleomorphic cell pavement. Phase contrast. Original magnification x 150

TC 23. Anaplastic carcinoma, partly intraduct. 7 days culture.



TC 24. Intraduct carcinoma with considerable invasion.
23 days culture.

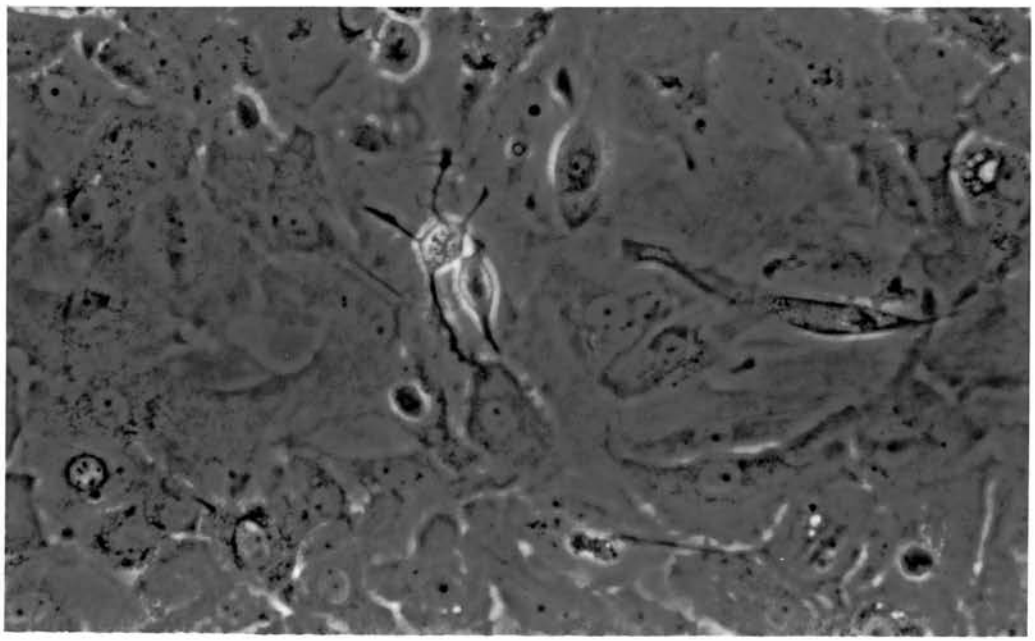


Fig. 239.

Indistinct cells with cellular projections. Phase contrast.

Original magnification x 150

TYPE VII. "Indistinct Cells with Cellular Projections".

This type of cell was often very indistinct when viewed using phase contrast microscopy. The cells were comparatively large and were well spread on the flask floor while their cell membranes and nuclei were very difficult to distinguish. The cells were further characterised by the presence of cellular material projecting from the cell pavement up into the supernatant medium. Detailed study of the projections revealed that they were flattened complete cells which had been "released" from the flask floor. This type of cell was fairly rare but occurred in cultures from both neoplastic and non-neoplastic tissues.

TC 52. Highly elastotic anaplastic carcinoma. 6 days culture.

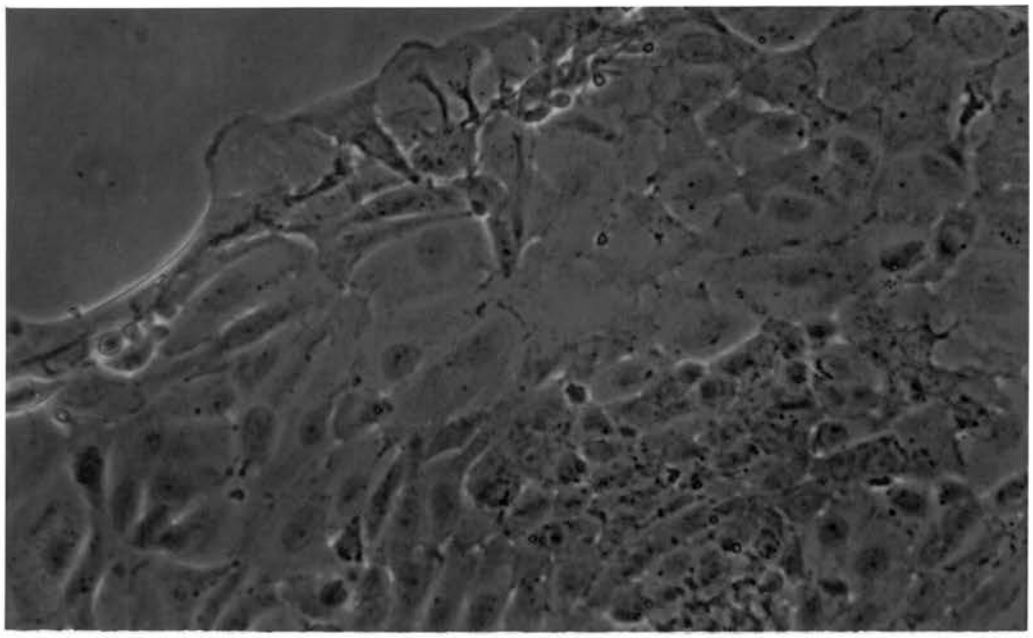


Fig. 240.

Indistinct cells with cellular projections. Phase contrast.

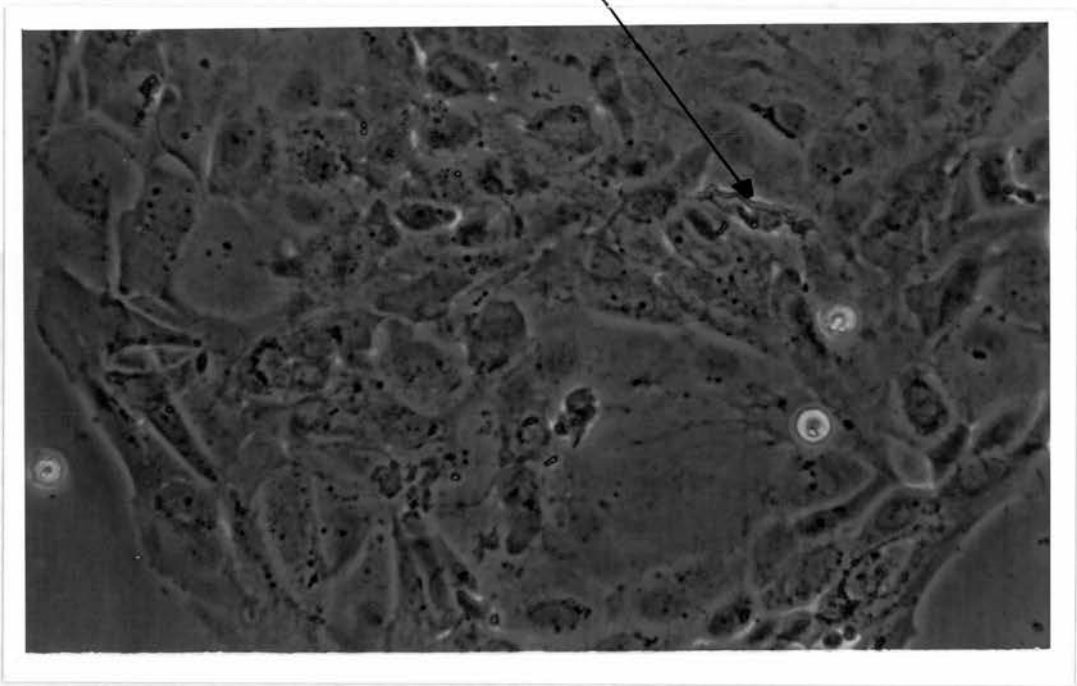
Original magnification x 150

Fig. 241.

Indistinct cells with cellular projections. Phase contrast.

Original magnification x 150

TC 52. Cellular projection.



TC 23. Anaplastic carcinoma, partly intraduct. 29 days culture.

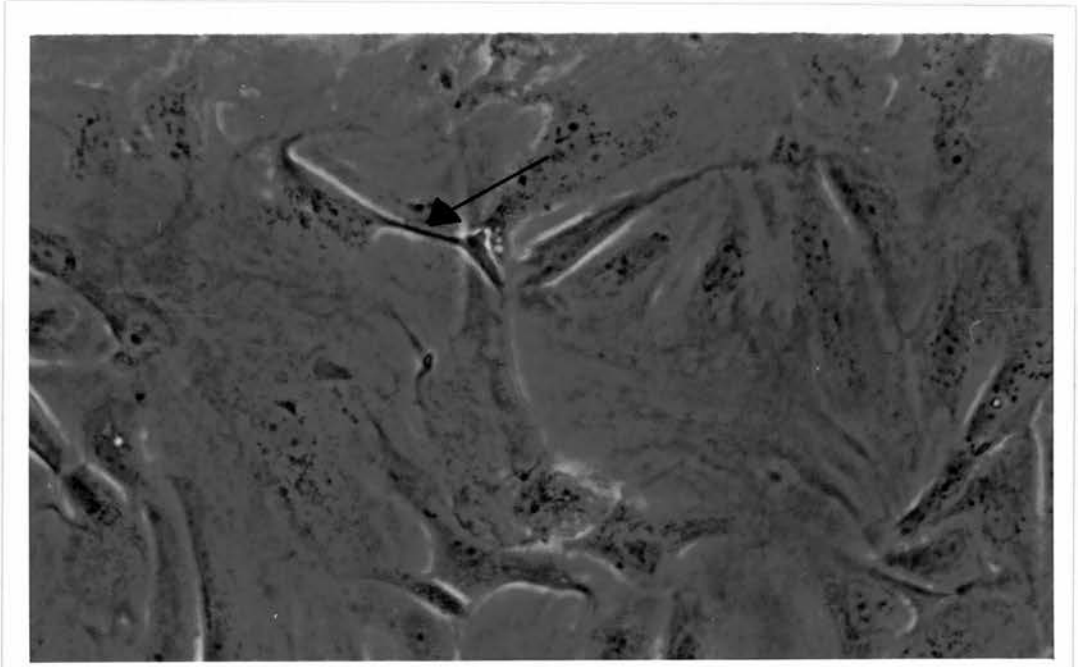


Fig. 242.

Cellular projection on a higher plane of focus. Phase contrast.

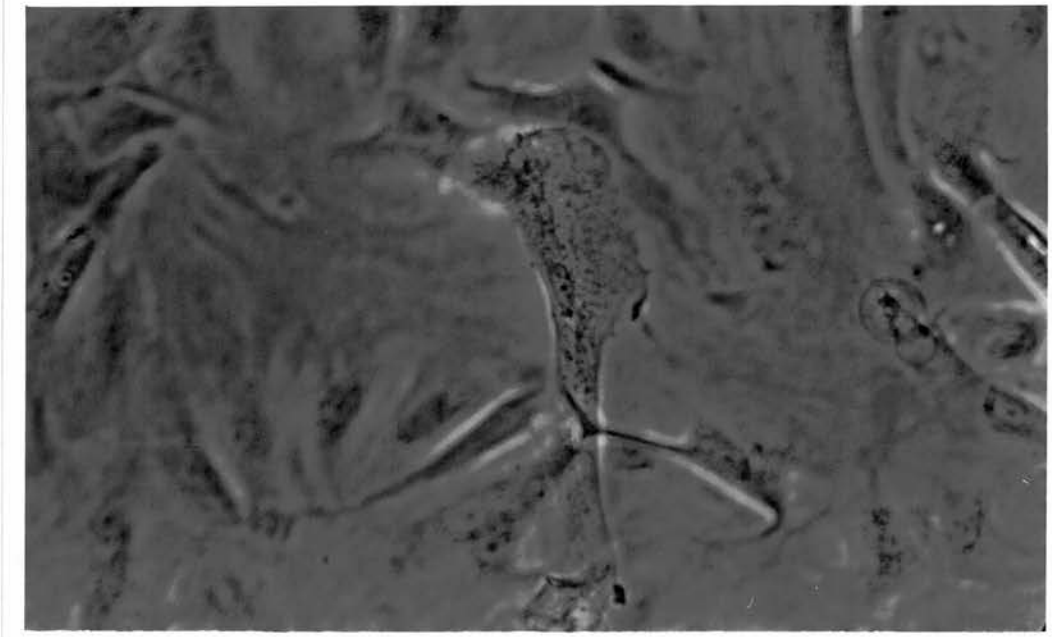
Original magnification x 150

Fig. 243.

Indistinct cells with cellular projections. Phase contrast.

Original magnification x 150

TC 23. Detail of projection, revealing its identity as a cell.



TC 84. Anaplastic carcinoma of large cell type. 20 days culture. An area containing many cellular projections.

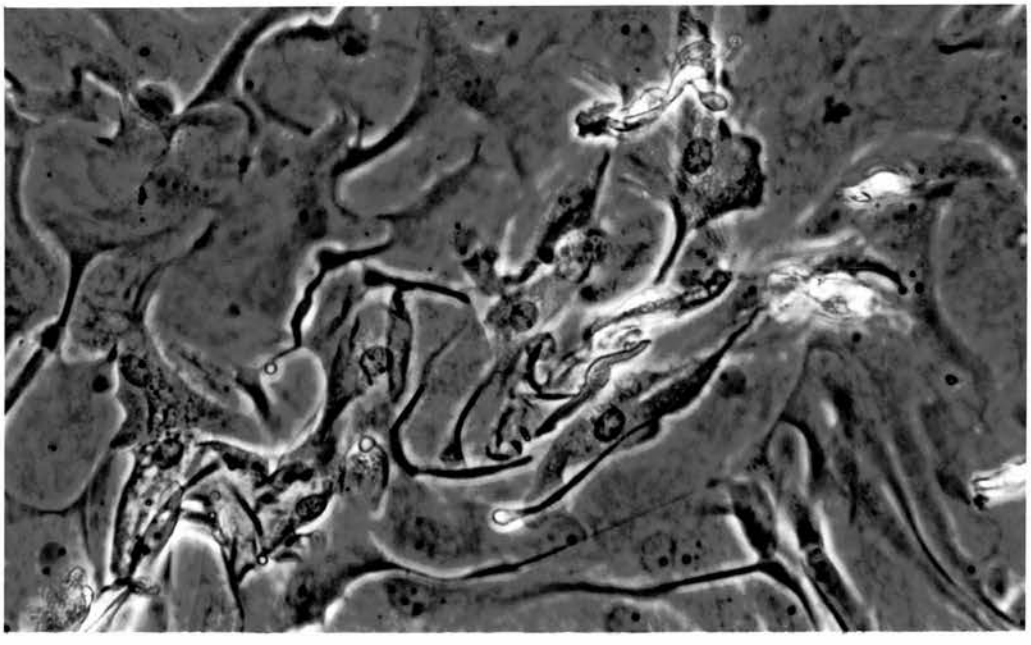


Fig. 244.

TC 84.

A detailed view of one of the cellular projections.

Phase contrast. Original magnification X 300.

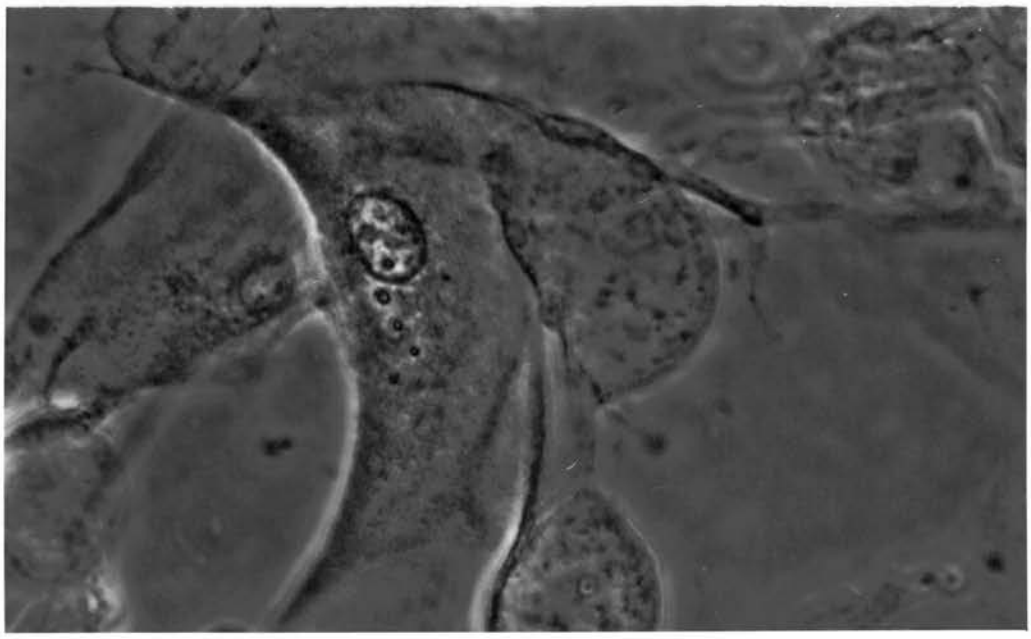


Fig. 245.

Round cells. Phase contrast. Original magnification x 150

TYPE VIII. "Round Cells"

Round cells were large, almost circular cells with very extended cytoplasm. The nucleus was round or oval and pale with one or two nucleoli. There was often a granular zone around the nucleus and the cells sometimes appeared to have particulate "matter" attached to their upper surface. These cells are probably analogous to the "Mesothelial-like" cells described by Relda Cailleau [26] and found by her and her co-workers in cultures established from pleural effusions. As with the cultures described by Relda Cailleau, Round cells showed no evidence of cell division for the duration of the culture period. They were found in all types of culture.

TC 76. Highly elastotic anaplastic carcinoma. 30 days culture.

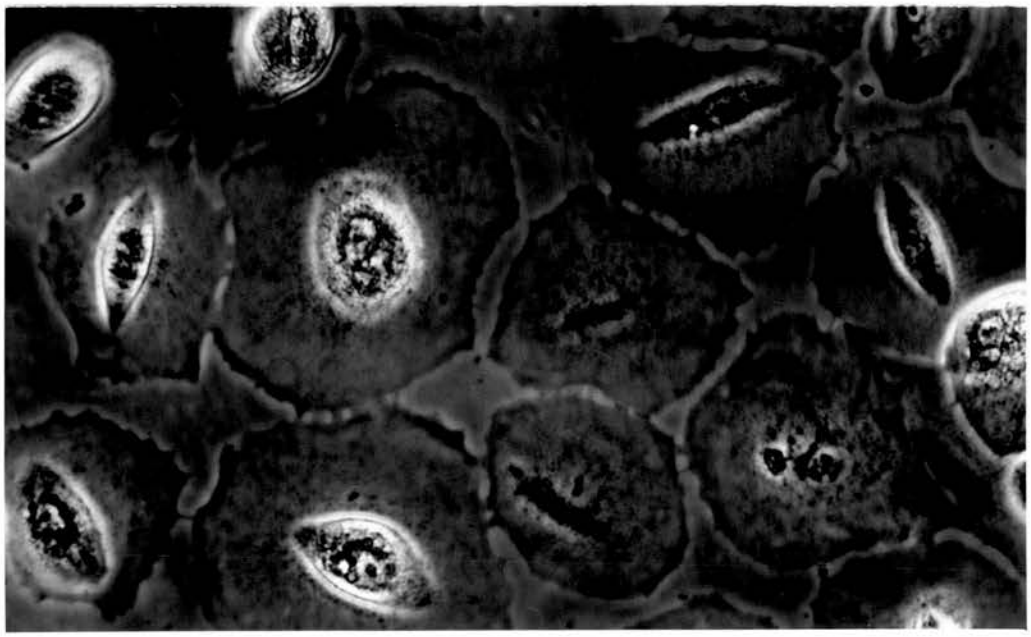


Fig. 246.

Round cells. Phase contrast. Original magnification x 150

TC 23. Anaplastic carcinoma, partly intraduct. 12 days culture.

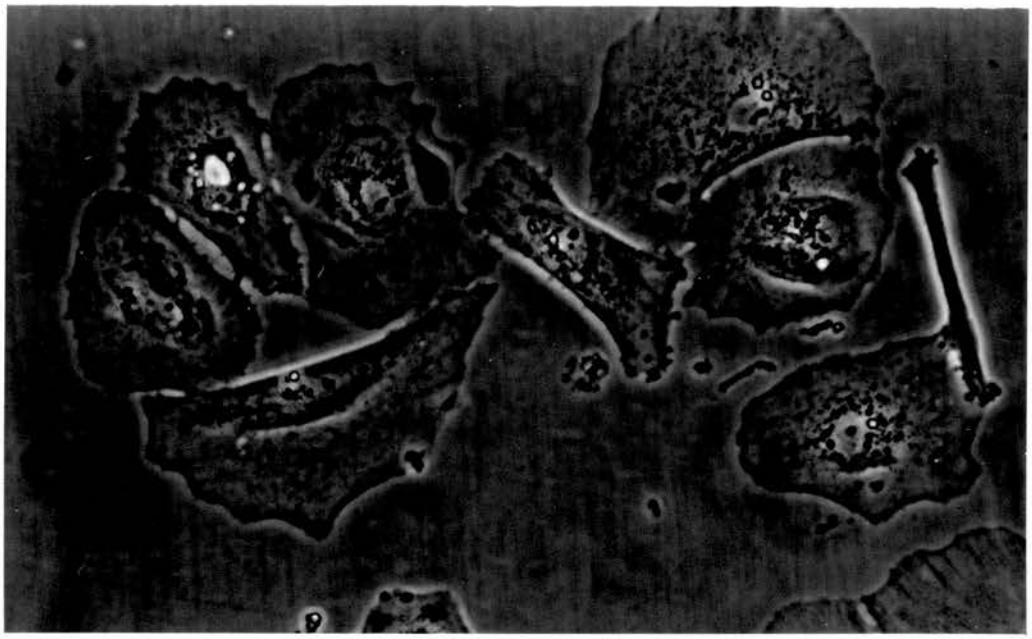


Fig. 247.

Elastotic cells as depicted previously in Fig. 3. (p 49). Phase contrast.

Original magnification x 150

TYPE IX. "Elastotic Cells".

Elastotic cells were described fully in Section I (p. 48). These were cells of variable shape whose characteristic feature was the persistent "blebbing" of cellular material from the surface of the cell. Elastotic cells were found almost exclusively in cultures from tumours which contained a significant proportion of tumour elastica.

TC 67. Lobular carcinoma with a high degree of elastosis.

7 days culture.

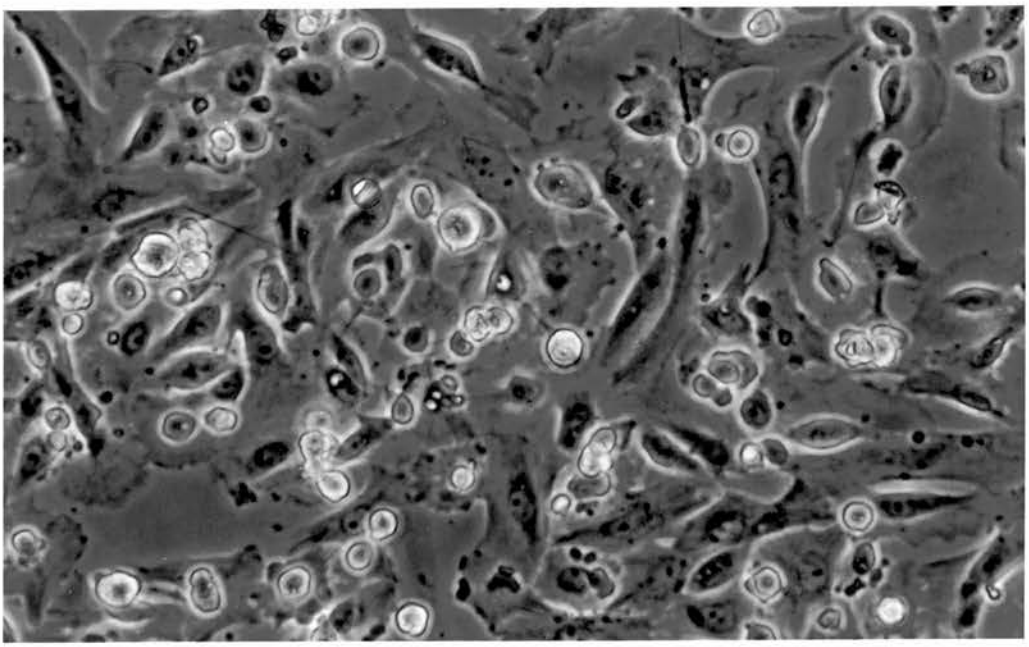


Fig. 248.

'Giant cells'. Phase contrast. Original magnification x 150

TYPE X. "Giant Cells".

Giant cells were noted in only four cultures and were so classified on the presence of multiple nuclei in a very large cytoplasmic mass. They were found once in a non-elastotic tumour culture, twice in cultures from elastotic tumours and once in a culture from a case of fibrocystic disease. Other workers have recorded the presence of multinucleate giant cells in culture. Coman[33] recorded their presence in cultures of benign epithelial tumours, and Das[37], reported the presence of cells with as many as eight nuclei in cultures of mouse mammary tumours. The significance of this type of cell is not known.

TC 76. Highly elastotic anaplastic carcinoma. 57 days culture.

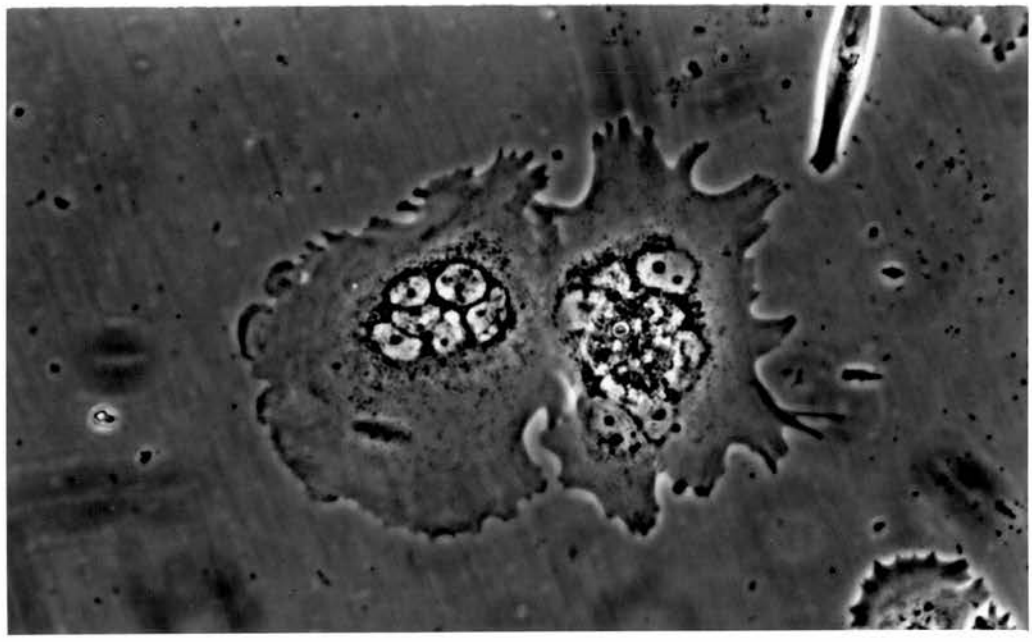


Fig. 249.

'Giant cells'. Phase contrast. Original magnification x 150

TC 12. Fibrocystic disease with considerable adenosis.

5 days culture.

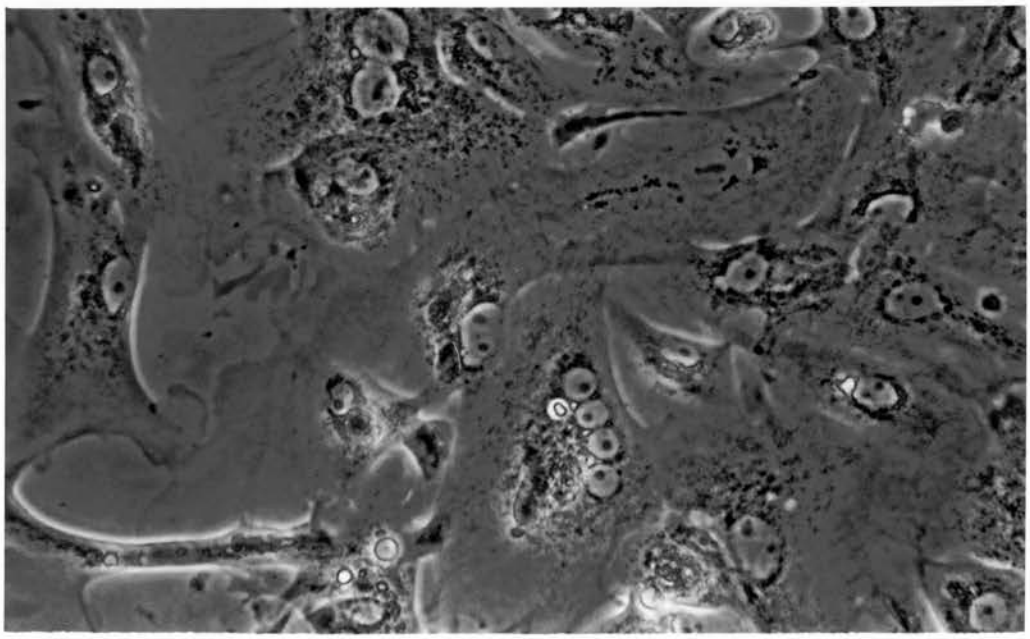


Fig. 250.

'Fairy' cells. Phase contrast. Original magnification x 150

TYPE XI. "Fairy Cells"

In the present series of cultures, fairy cells were found exclusively in cultures of malignant tissue. The cells settled singly on the flask floor early in the culture period and from a very "shiny" central cell body several arm-like processes extended. The nucleus was difficult to resolve and, in many cases, the cells did not appear to divide but remained virtually static for the duration of the culture.

The fairy cells which grew in the present series of cultures are identical in appearance to the "dendritic cells" described by Relda Cailleau [26] and found by her in cultures of malignant pleural effusions. The function of these cells is not known.

TC 57. Anaplastic carcinoma. 2 days culture.

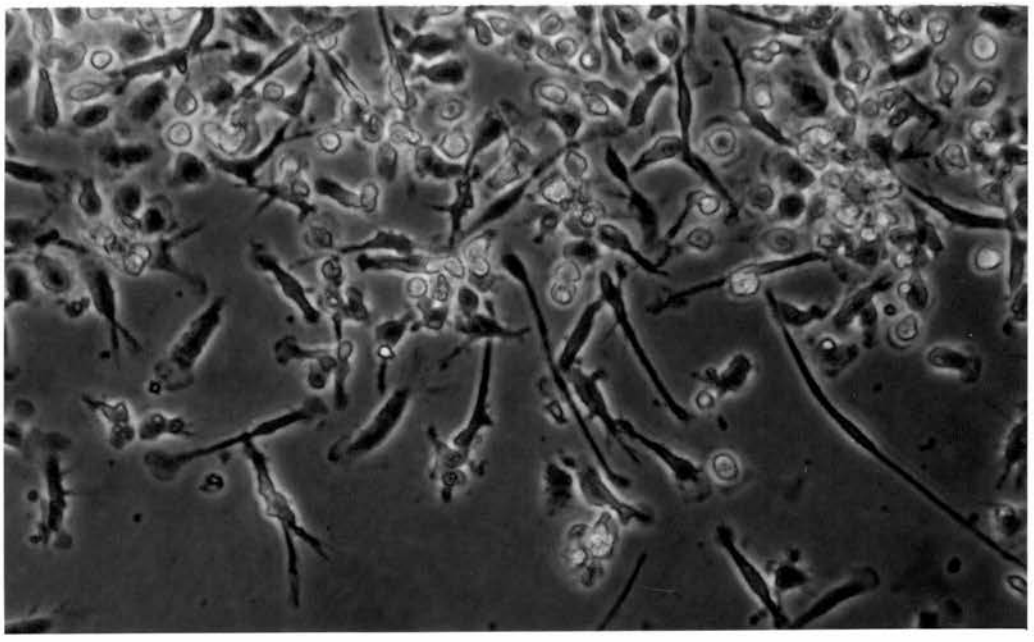


Fig. 251.

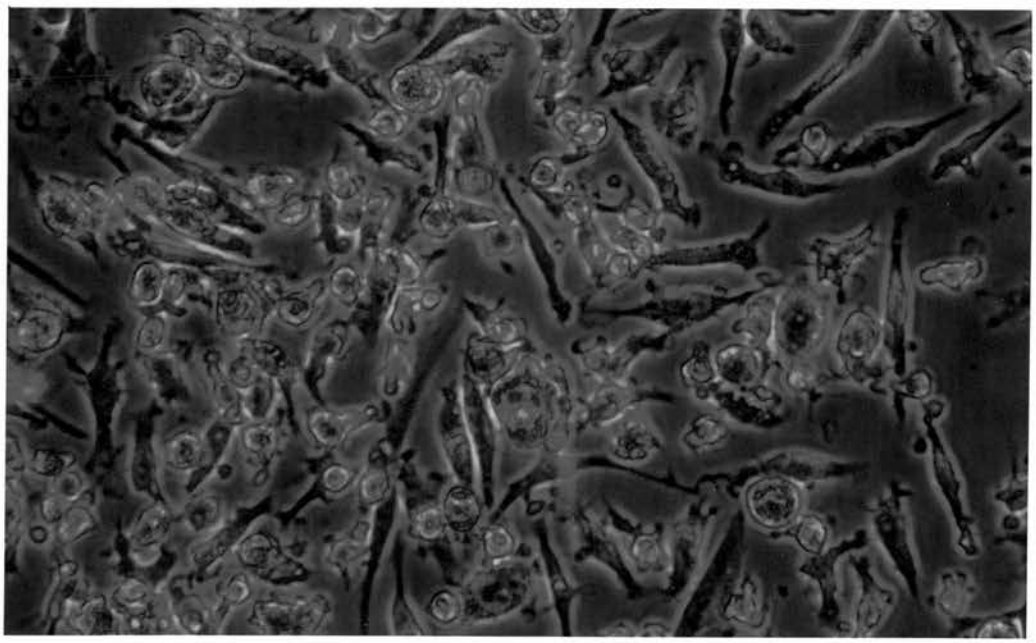
'Fairy' cells. Phase contrast. Original magnification x 150

Fig. 252.

'Fairy' cells together with round cells. Phase contrast.
Original magnification x 150

TC 53. Anaplastic carcinoma, little "stromal reaction".

5 days culture.



TC 74. Anaplastic carcinoma with tracts of necrosis.

5 days culture.

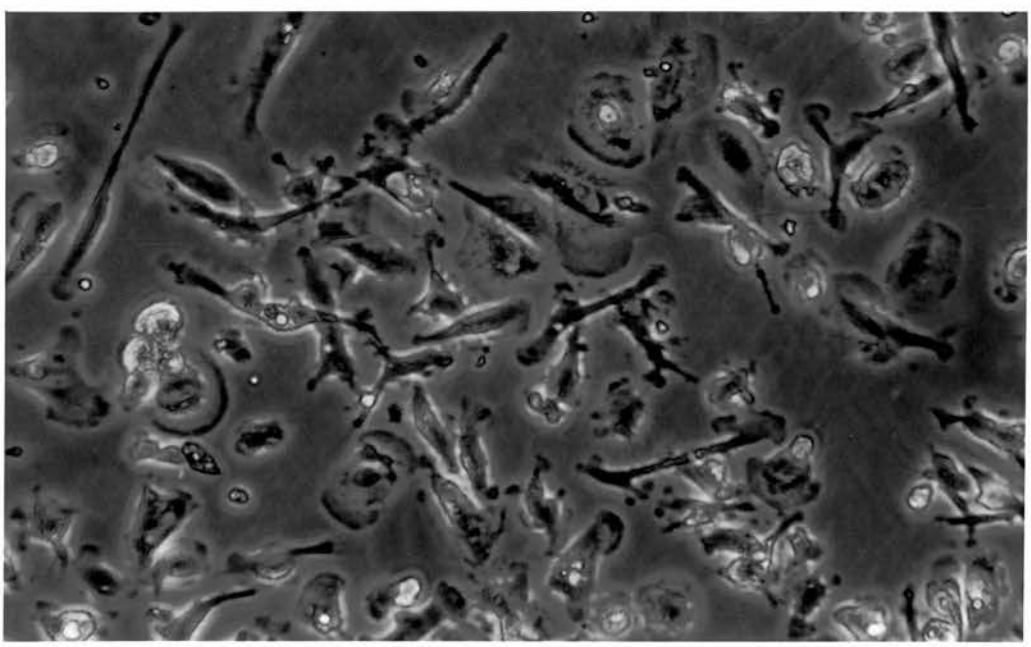


Fig. 253.

'Fairy' cells. Phase contrast. Original magnification x 150

TC 67. Highly elastotic lobular carcinoma. 16 days culture.

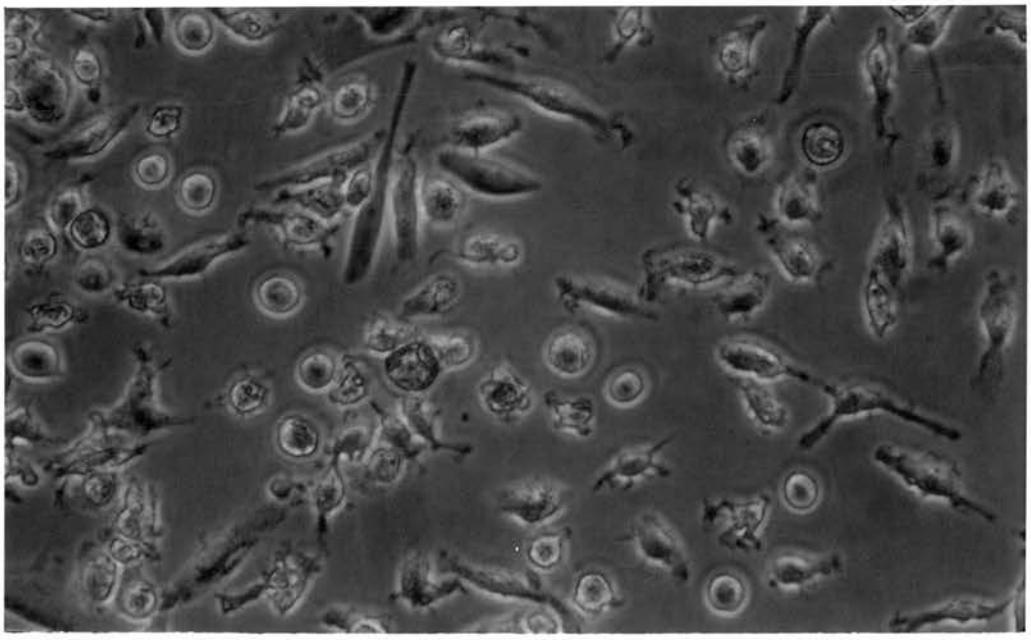


Fig. 254.

Cellular sphere. Phase contrast. Original magnification x 150

TYPE XII. "Cell Spheres"

Cellular spheres were described fully in Section III (p 143). They were characteristic of cultures of cells from tumours possessing an apocrine component. The cellular spheres were of many sizes and consisted of hollow balls of viable cells which floated in the culture medium for the duration of the culture.

TC 68. Highly elastotic anaplastic carcinoma with an apocrine component. 27 days culture.

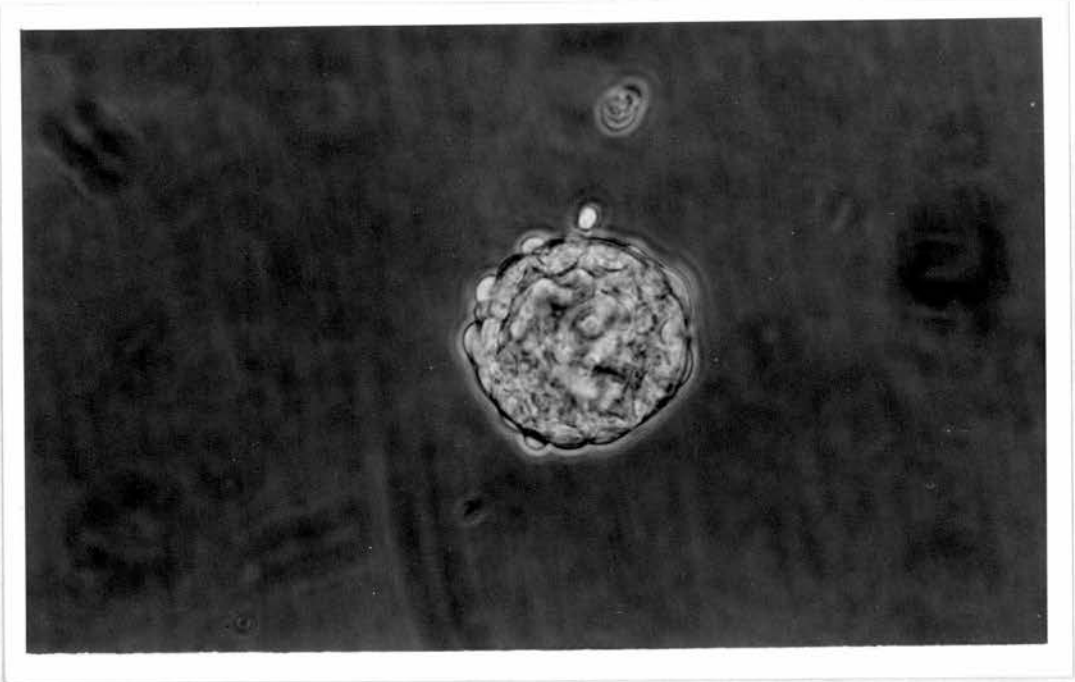


Fig. 255.

'Globules' are depicted in Fig. 179 (p 165). Phase contrast.

Original magnification x 150

TYPE XIII. "Globules"

Globules were described fully in Section III (p. 143). The globules were refringent collections of material which accumulated between cell layers in cultures of tissue from tumours with an apocrine component. The globules were thought to be composed of mucopolysaccharide (p. 165).

TC 32. Anaplastic carcinoma. 15 days culture.

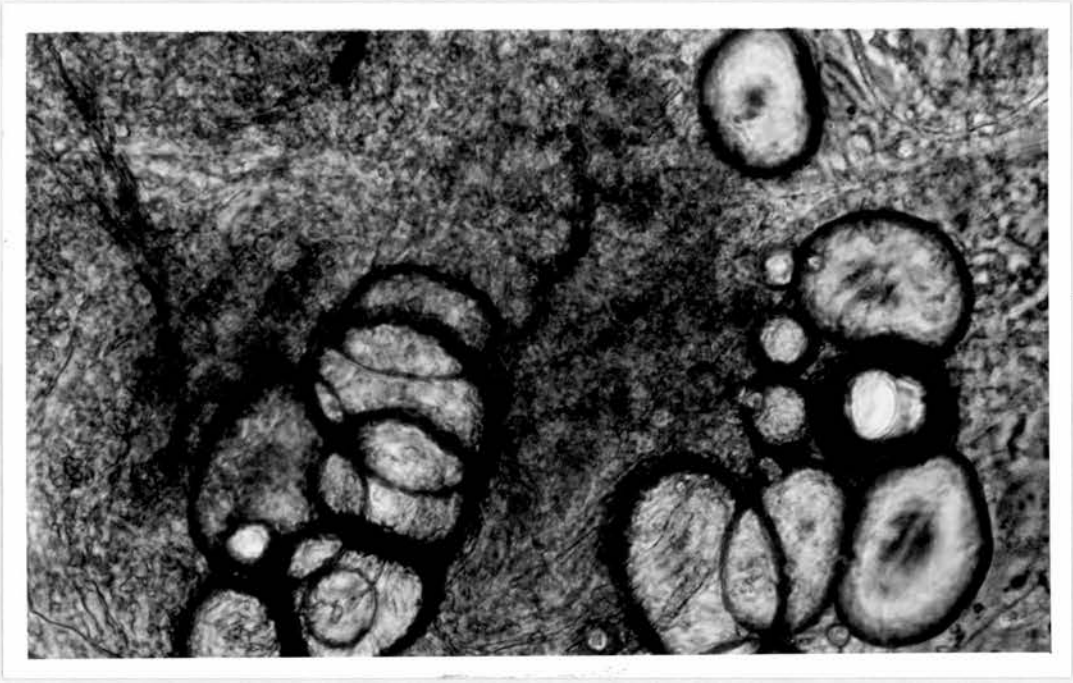


Fig. 256.

Fibroblasts. Phase contrast. Original magnification x 150

TYPE XIV. Fibroblasts.

As many other workers in the field of tissue culture have found, the growth of fibroblasts was a common occurrence in cultures of breast tissue. In many cases the fibroblasts migrated from the small lumps of tissue which were transferred to the incubation flasks with the original cell inoculum. Those cells which have been classified as fibroblasts in this study were long, thin cells which lined up in parallel rows and also tended to pile up into multiple layers. The distinction drawn between fibroblasts and myofibroblasts (Type XV), on the basis of light microscopy, is to some extent arbitrary.

TC 76. Highly elastotic anaplastic carcinoma. 30 days culture.

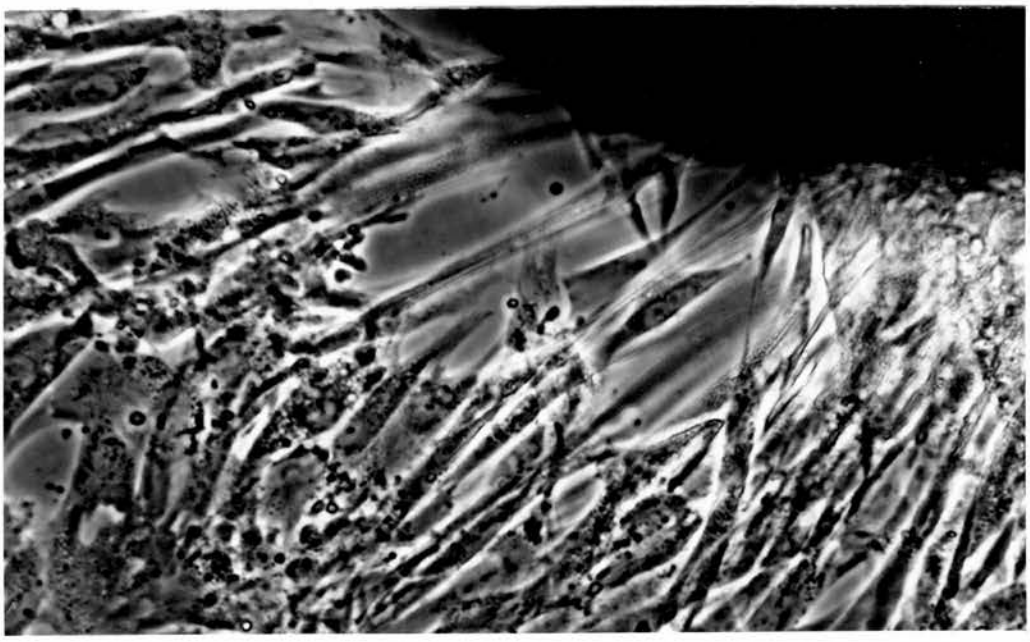


Fig. 257.

Fibroblasts. Phase contrast. Original magnification x 150

TC 63. Medullary carcinoma with scirrhous areas.

30 days culture.

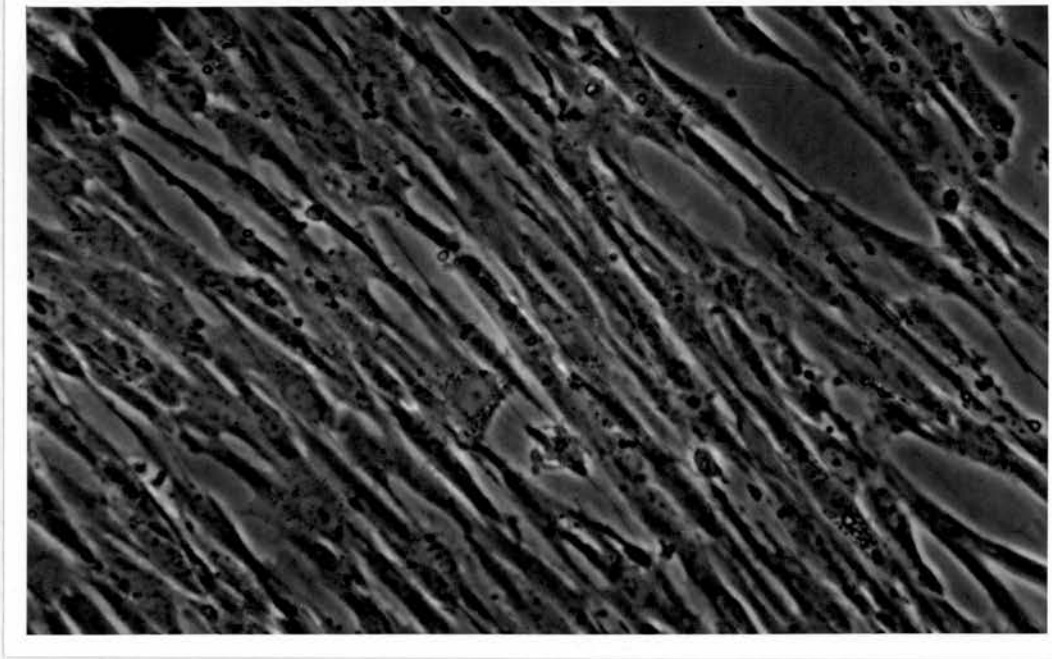


Fig. 258.

Myofibroblasts. Phase contrast. Original magnification x 150

TYPE XV. Myofibroblasts.

Myofibroblasts were described fully in Section I (p. 99). Myofibroblasts were classified according to their property of contraction. Initially the cells spread out on the flask floor with many cytoplasmic processes and slightly granular perinuclear cytoplasm. The nuclei were oval, pale and contained several prominent nucleoli. Division was fairly rapid and as the number of cells increased they piled up on one another in refringent ridges. Periodically the sheets and ridges of cells appeared to contract and "lumps" of tissue were formed. The property of contraction was retained when the cells were passaged (see p. 100), as was the ability to form a variety of connective tissue elements (see pp 101).

TC 61. Scirrhous and medullary carcinoma. 51 days culture showing refringent cell ridge.

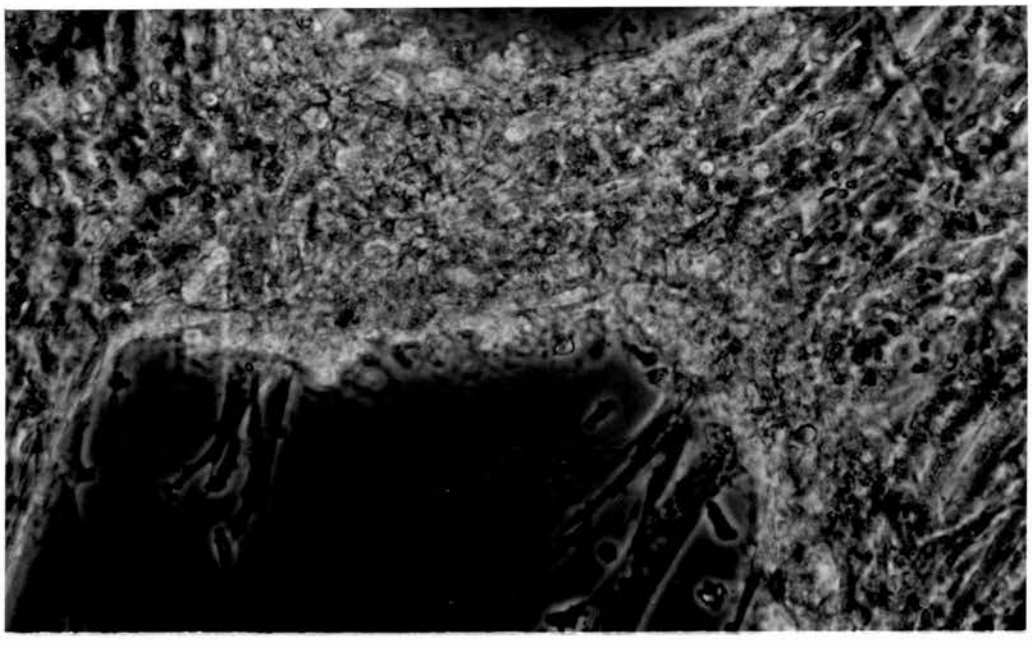


Fig. 259.

Phase contrast. Original magnification x 150

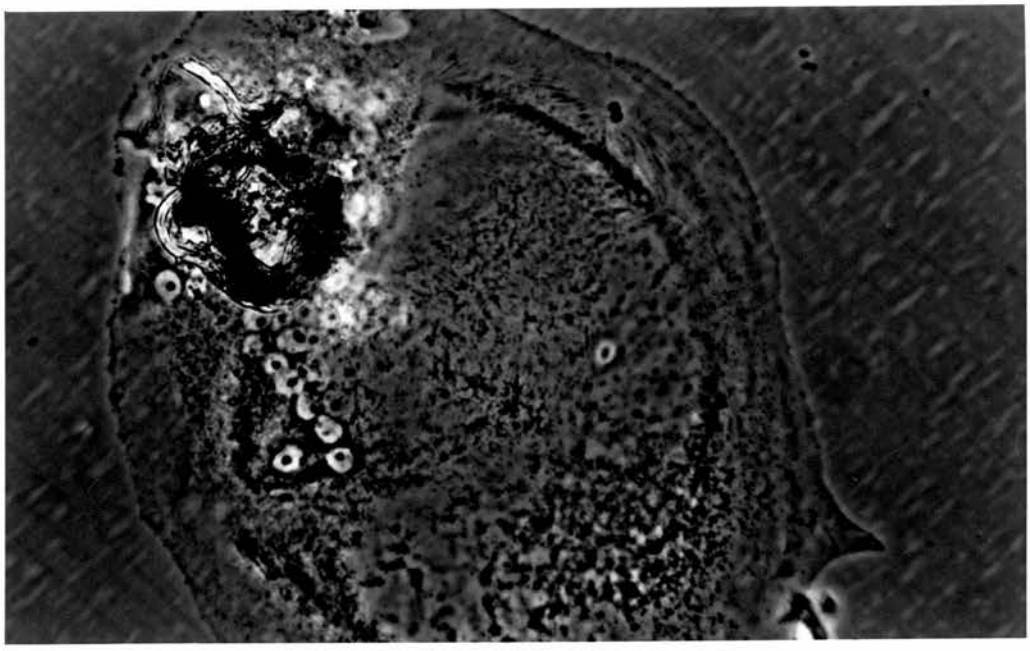
Fig. 260.

Phase contrast. Original magnification x 300

TYPE XVI. Unidentifiable Cells.

There were several cells which appeared during the course of the study which defied classification and which are included here to complete the survey of the cell types grown. It is probable that, in many cases, the cells depicted are senescent.

TC 82. Highly elastotic anaplastic carcinoma. 14 days culture.



TC 67. Highly elastotic lobular carcinoma. 2 days culture.

X 300

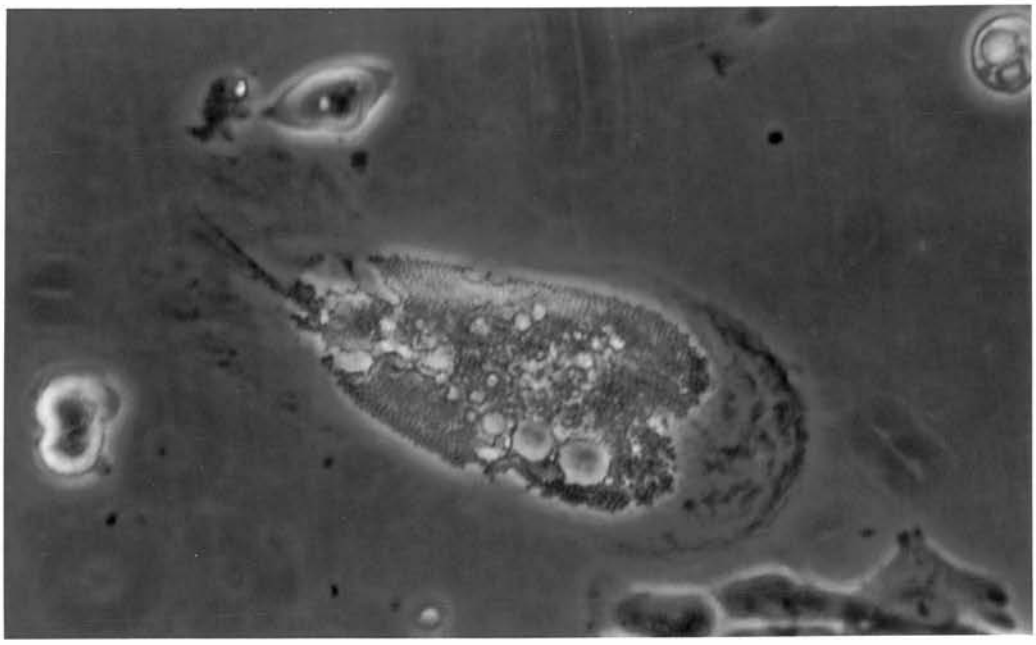


Fig. 261.

Phase contrast. Original magnification x 300

Fig. 262.

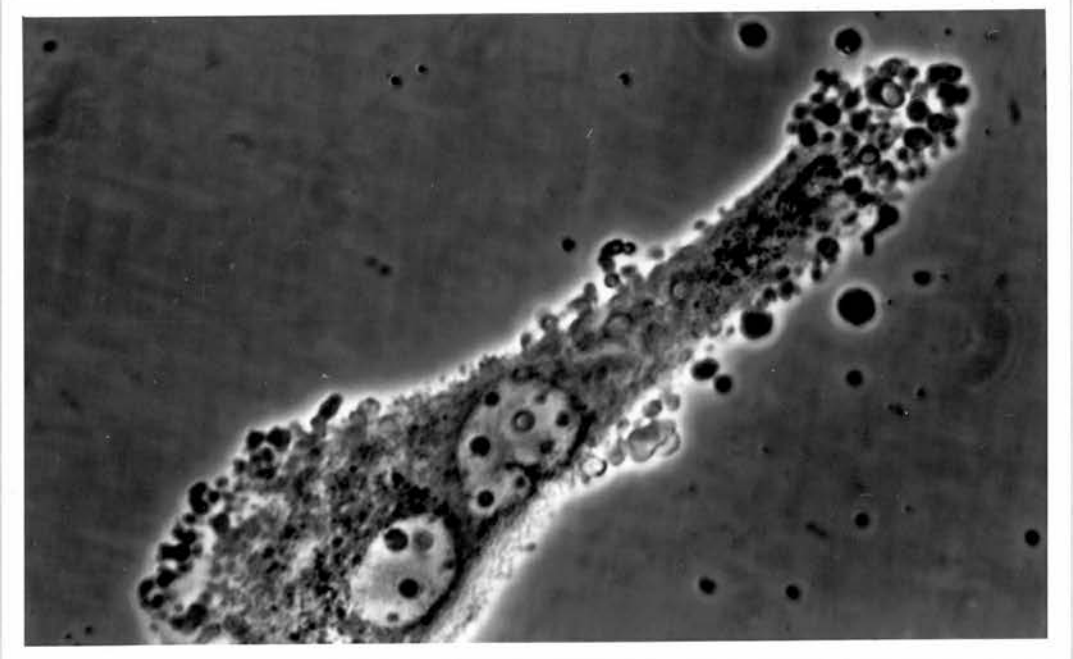
TC 85.

Phase contrast. Original magnification x 300.

TC 85. Anaplastic carcinoma of large cell type.

56 days culture.

As above. 63 days culture. Original magnification X 300



GENERAL DISCUSSION

GENERAL DISCUSSION

For many years before the initiation of the present study, workers had cultured human breast cancer cells, albeit with a limited degree of success. None, however, directed their attention towards the differences in cultural characteristics which might exist between those tumours containing only a moderate amount of elastosis, or none at all, and those assigned an elastica index[150] of II, the type of carcinoma shown in 1972 to be associated with a relatively good prognosis.[105, 150, 172] It was on this aspect that this work was originally focussed; the first task being to grow, in culture, cells from a tumour which was known to be highly elastotic.

The disadvantage of any study of this type is that, although cells may grow from the tumour tissue, there is no guarantee that the surviving, replicating cells are those which are associated with the feature in which the interest lies - in this case elastosis. However, the recognition in this investigation of cell types which were apparently intimately associated with the production of elastin lends support to the conclusion that it was, indeed, the particular cells of interest which were growing. The belief that it is the neoplastic epithelial cells in a breast tumour which produce some, if not all, of the elastin present within the tumour also receives support.

The origin of elastin in breast tumour tissue has been attributed both to the neoplastic epithelial cells and to the, so-called, 'stromal' fibroblasts.

As long ago as 1957 Jackson and Orr[79] put forward the idea that collagenosis and elastosis were not a reaction to the neoplastic epithelium but were: "changes developing 'pari passu' with the malignant change in the epithelium and sometimes preceding it". Douglas and Shivas[41] concluded/

concluded that the origin of the elastin was within the tumour cells, on the basis of both the morphological position of the pericellular elastin and on evidence from transmission electron microscopy. This view was strongly supported by the immunohistochemical studies of Al-Adnani, Kirrane and McGee[4]. They found prolyl hydroxylase, an enzyme essential to the production of another connective tissue protein, collagen, together with collagen itself, in carcinoma cells of thirty out of thirty two scirrhous carcinomas examined. No collagen or prolyl hydroxylase was found in the spindle cells in the stroma.

McCullagh,[109] also employing immunohistochemical techniques, has provided the most convincing evidence for the tumour cell origin of elastin by demonstrating the binding of a highly purified antibody to elastin to tumour cell surfaces but not to the adjacent stromal fibroblasts.

The opposing view, that it is the non-neoplastic 'stromal' fibroblasts which produce the tumour elastin, has been put forward by Azzopardi[8] and is weakly supported by the observations by Tremblay[162] of 'active' fibroblasts i.e., cells containing abundant rough endoplasmic reticulum, prominent Golgi complexes and bundles of filaments, adjacent to the tumour elastin. However, there was no recognisable elastin associated with the stromal fibroblasts.

The results obtained in the present study suggest that the elastin is produced by both neoplastic epithelial cells, via the 'blebbing' phenomenon, and, through some alternative mechanism, by cells recognised as myofibroblasts.

A criticism of any study which relies solely on a histological staining technique for the demonstration of a defined chemical entity relates to the specificity of that particular stain. Gomori's aldehyde fuchsin staining technique,[62] which was used for these experiments, stains/

stains sulphated and neutral mucosubstances and glycogen, in addition to coarse and fine elastic fibres. Confirmation of the identity of the material, which apparently stained positively for elastin, is therefore required and the use of biochemical or immunological techniques is indicated.

Two types of elastin have been described in breast tumours,[8] the so-called focal deposits which are said to occur around pre-existing ducts, veins and arteries, and the diffuse form which appears as single or groups of fibres distributed in the 'stroma'.

It is tempting to suggest that the two forms of elastin identified here represent these two types of deposit and that the reason for their apparent differences in structure and distribution lies in their different points of origin. It is conceivable that the focal deposits of elastin might be produced by the neoplastic epithelial cells and that the diffuse, fibrillar elastin originates in the myofibroblasts.

The suggestion that myofibroblasts might be implicated in elastosis was made originally by Seemayer et al[149], based on a combination of the presence of myofibroblasts in breast cancers and evidence provided by work on the aorta which associated the myofibroblast with elastin synthesis[178]. Ahmed[2], by inference, had also suggested this point of origin, in 1974, but had not recognised the cell which he thought differentiated from myoepithelial cells, a normal component of the mammary lobular structure[124], as a myofibroblast.

The apparent demonstration in this experimental work of active elastin synthesis in culture by cells which, on behavioural and ultrastructural grounds, were myofibroblasts, would seem to suggest that myofibroblasts do contribute at least some of the elastin in highly elastotic breast tumours.

There/

There immediately arises the question of whether the myofibroblasts present in the breast tumours are neoplastic (i.e. tumour cells) or occur as part of some 'stromal reaction' mounted against the epithelial tumour cells.

Seemayer[149] maintained that, although myofibroblasts are not normally present in mammary stroma, they are a non-neoplastic component of the 'stromal reaction' arising by modification of normal stromal fibroblasts. This view was supported by Ohtani and Sasano[118, 119].

The alternative view, that myofibroblasts can undergo malignant change, has been put forward by two groups of workers, led by Churg[31] and Vasudev[166], based on their ultrastructural observations of fibrosarcomas and fibrous histiocytomas.

This view was supported indirectly by McCullagh et al[109] who, although not making any mention of the presence of myofibroblasts, alluded to the observations of Murad and Scarpelli[113], who found numerous intracytoplasmic myofibrils in the malignant cells of mammary carcinomas. McCullagh suggested that "elastogenesis may be a feature of cells with myofibrillar differentiation and that the capacity to synthesise elastic tissue is maintained in mammary epithelial cells and expressed following malignant transformation in scirrhous carcinoma".

That the myofibrillar cells are myofibroblasts and not myoepithelial cells remains to be confirmed. In the present study no typical desmosomes, features taken to be characteristic of normal myoepithelial cells, were found between the cells in question. However, a characteristic of neoplastic transformation is the loss of 'normal' structure, so it may be that the normal myoepithelial cells bounding the terminal acinar structures of the breast 'transform' and become capable of elastin synthesis. This view was put forward by David and Buchner[38] in their recent/

recent electron microscopic investigation of elastosis in benign and malignant salivary gland tumours. Their evidence strongly supports the hypothesis that myoepithelial-like tumour cells produce the elastin in this situation.

The biological status and inter-relationship of myofibroblasts, myoepithelial cells and epithelial cells 'in situ' in a tumour thus remains equivocal.

Myofibroblasts, in culture, tended to pile up on one another in multiple layers and this feature is taken to be indicative of the occurrence of neoplastic transformation[1, 161]. The possibility that this loss of contact inhibition might indicate an infection with mycoplasma is considered unlikely since mycoplasma-induced growth stimulation has only been reported in short term lymphocyte cultures[16] and the usual effect of an infection of this type is to inhibit growth[157].

Therefore, the inference is that the myofibroblasts cultured were, indeed, neoplastic or pre-neoplastic and that the elastin in mammary tumours is predominantly tumour cell derived.

If this is the case, then the focal deposits described by Muir and Aitkenhead[112] are not laid down by normal fibroblasts around pre-existing structures (except in so far as some elastosis does occur around ducts in fibrocystic disease) but represent an anomalous differentiation caused by the activity of neoplastic cells, as Shivas and Tansey[153] concluded. As tumour cells may be found scattered throughout the tumour stroma[109], focal and diffuse forms of elastin may therefore be merely different manifestations of the same tumour cell mediated phenomenon.

Further support for the 'anomalous differentiation' theory comes from consideration of the distribution of vascular structures and ducts within mammary carcinomas of elastosis grades 0 and I. Within these tumours/

tumours veins, arteries and pre-existing ducts are uncommon so that, even accepting Jackson and Orr's[79] concept of 'a collapse of the mammary duct system', it seems unlikely that enough pre-existing structures would be present to account for the very numerous focal deposits of elastin often seen in grade II tumours. Therefore, unless there is an enormous increase in normal vascular elements in a tumour of elastosis grade II, the tubes and cords of elastin must arise by 'de novo' synthesis and random distribution, as Shivas and Tansey[153] maintained.

If the concept of mammary duct collapse is untenable, the explanation of the apparent reduction in the volume occupied by a tumour, as compared with that of the pre-existing normal mammary tissue, must lie elsewhere. This study has indicated that myofibroblasts play an active part in the biology of a breast tumour. It is possible, therefore, that the apparent reduction in volume produced by the tumour tissue may be caused by contraction of myofibroblasts, in a similar manner to that observed in wound contraction[55].

The belief that connective tissue proteins may originate in neoplastic cells was further corroborated by the demonstration of both pericellular and fibrillar reticulin in cultures of breast tumour cells.

It is generally thought that reticulin, possessing the same axial periodicity as collagen[91], is an immature form of collagen and, as such, is produced by fibroblasts. Certainly there has been little experimental evidence to suggest that any other cell might be responsible for its formation.

In the present study, as with the synthesis of elastin, myofibroblasts were implicated in the production of the fibrillar material. The source of the pericellular deposits of reticulin found around the epithelial cells seems likely to be the epithelial cells themselves as, although epithelial cells at the periphery of the cell groups were often spindled, fibroblastic growth/

growth was absent from those tumour cultures in which the pericellular reticulin was observed.

The phenomenon of peripheral spindling has been noted before[47, 84, 163, 164] and has been attributed to the mechanical constraints placed upon cells in a liquid[164] or semi-solid[47] medium. The fact that the coincident halo which often occurs with peripheral spindling equates with the presence of reticulin is, apparently, a new observation. In view of the capacity of these cells to produce connective tissue proteins it seems possible that the observed alteration in cell shape is merely an expression of the change in function of the cell, the more 'fibroblastic' the cell function becomes, the more spindled its shape.

The idea that the reticulin found in breast tumours might be tumour cell derived has been postulated before. Gonzales-Licea et al[64], while reporting on the ultrastructure of a malignant breast tumour with bone formation, commented that "fine reticular fibres were observed, with the silver reticulin stain, between almost every undifferentiated tumour cell". They later went on to say that "collagen fibrils (in the electron micrographs) corresponded to the reticulin seen by silver staining" and they concluded that the production of connective tissues "is not inconsistent with epithelial origin".

A similar phenomenon to that seen in epithelial mammary tumour cell cultures may be observed in the liver primordium of a developing embryo. Primitive hepatocytes, of undeniable epithelial origin, can clearly be seen to be 'spindling' and application of Gordon and Sweets' silver impregnation technique reveals pericellular reticulin around each primitive liver cell. (A.A. Shivas - personal communication).

Fine reticulin fibres have long been known to be associated with basement/

basement membranes[17] and immunofluorescent studies conducted in 1963[128] suggested that epithelial basement membranes are produced by epithelial cells. More recently Liotta et al[94, 95, 96], in a series of very elegant experiments, demonstrated both the production of basement membrane type collagen by breast tumour cells and, of immense potential use diagnostically, the location of the same type of collagen around unicellular metastatic deposits in regional lymph nodes.

The term 'stromal reaction' used to describe the connective tissue in a tumour would thus appear to be a misnomer as a large proportion of the tumour connective tissue is, apparently, produced by the tumour cells themselves. This view does not, however, preclude the contribution of connective tissue elements by non-neoplastic cells in the vicinity of the tumour.

The foregoing evidence suggests a need to reappraise the rigid compartmentalisation of cells into 'connective tissue producing fibroblasts' on the one hand and 'non connective tissue producing epithelial cells' on the other. There is a definite indication that a spectrum of cell form and function exists, the two extremes of which are those cited.

This view would help to explain the wide range of cell types recognised in this investigation and the reason for the difficulties which were often encountered when assigning a cell type to a particular category. The tissue culture situation provides an artificial environment for the growing cells which 'in vivo' would be influenced by a much more complex environment. It may be that, in this artificial situation, the often minute differences in cell morphology and behaviour are given a greater opportunity for expression.

A good illustration of this is the demonstration, in tissue culture, of functioning apocrine cells. The presence of cells of apocrine type in/

in a mammary carcinoma is a relatively common finding but it is rarely reported as its clinical significance is thought to be negligible[107]. In routine histological sections of apocrine carcinomas, although cells bordering a duct lumen may show characteristic 'snouts'[70], the plane of section will often miss the duct lumen and so there is no proof that apocrine secretion is taking place. In the tissue culture situation the tumour cells are artificially provided with a free surface and active apocrine secretion may be observed.

Objections have often been raised to results obtained from tissue culture experiments, the critics claiming that observations made 'in vitro' may bear no relationship to what happens 'in vivo'. The demonstration of functioning apocrine cells in tissue culture, in addition to the observation of peripheral spindling with reticulin formation, would seem to vindicate the use of the technique suggesting that events 'in vitro' closely parallel what occurs 'in vivo'. The demonstrations also emphasise the value of tissue culture as a biological research tool.

Opinion regarding the significance of the presence of apocrine-type cells in mammary neoplasms is divided. Azzopardi[8] maintains that "the finding of apocrine foci is sufficiently useful as a confirmatory sign that a papillary tumour is benign" that it warrants extensive investigation to try to locate cells of this type within this uncommon neoplasm. In believing that apocrine cells are not pre-malignant he supports the views of Dawson[36] who felt that apocrine or 'pale' epithelium represented a degenerative change which ended in the formation of quiescent cysts, not malignant lesions. Haagensen[70], on the other hand, devotes a small section of his book to malignant 'apocrine carcinomas' of the breast.

The/

The controversy which surrounds the issue of whether apocrine metaplasia is quiescent, pre-malignant or malignant, is probably a reflection of the relative rarity of tumours designated apocrine carcinomas. Wald[169] reported an incidence of 1-15%, depending on the rigidity of the criteria used for classification, and, in a sixteen year study, Frable and Kay[53] diagnosed only nineteen apocrine carcinomas, an incidence of 1%.

However, cells of apocrine type may be found in substantial numbers in most breast tumours. Indeed, Dawson[36] identified 'pale' cells in 116 out of 120 malignant and all non-malignant breast lesions studied. This frequency of occurrence is more in keeping with that found in these tissue culture studies (see Section III) and, perhaps, indicates that in the majority of cases the 'pale' cells as described by Dawson were grown.

The fact that apocrine cells may be grown and recognised in tissue culture might provide the means to determine the biological status of cells of apocrine type in mammary tissue.

The present study revealed a remarkable similarity in the light and electron microscope appearance of 'apocrine' cells and 'elastotic' cells. Both exhibited prominent 'blebbing' and, although in some cases the elastotic cells were elongated, it was sometimes impossible to classify a cell as one type or the other.

The possibility that the two cell types identified are, in fact, one cannot be ruled out. However, apocrine-type cells were identified in both mucoid carcinoma and in a male breast cancer, lesions in which elastosis is rarely a feature[153]. The distinctive 'blebbing' seen in both cell types may thus merely indicate that active secretion is taking place.

Since highly elastotic tumours are associated with a significant increase/

increase in survival, a question which is of crucial importance relates to the identity of the factor which triggers the synthesis and secretion of elastin.

An intriguing suggestion is that oestrogen may be involved. A recent paper by Westley and Rochefort[173] reported the secretion of a high molecular weight glycoprotein by three oestrogen receptor positive human breast cancer cell lines when the cells were stimulated with oestrogen. No glycoprotein synthesis occurred in an oestrogen receptor negative cell line under the same conditions.

A positive correlation has already been demonstrated between highly elastotic tumours and the presence of oestrogen receptors in breast tumours[105]. The likelihood that the, so far unidentified, glycoprotein synthesised is an elastin precursor thus seems high. It is interesting to note, in this context, that apocrine-type cells have been experimentally produced in the mammary glands of Rhesus monkeys[154] - also by the administration of oestrogen.

In summary it has been shown that cells from highly elastotic breast tumours can be grown in culture and are, apparently, capable of synthesising elastin. In addition, epithelial cells of tumour origin have been shown to exhibit peripheral spindling and coincident reticulin formation, a point hitherto unrecognised. These observations have been discussed in the light of current theories regarding the 'stromal reaction' to a tumour and the conclusion has been drawn that the connective tissue in a tumour is tumour-cell derived.

During the study cells of apocrine morphology were grown and functional apocrine secretion was observed. A similarity was noted between cells of apocrine and elastotic type and the possibility was raised that the characteristic secretory behaviour of these two cell types was oestrogen induced.

ACKNOWLEDGEMENTS

I should like to acknowledge all the people who have assisted me in the preparation of this thesis.

First I should like to thank Mr. Ian Capperauld and Ethicon Ltd. for making the study possible by financing the work.

To Dr. Andrew Shivas go my grateful thanks for his enthusiastic help and encouragement throughout the duration of the study and for his advice and criticism during the preparation of the manuscript.

Thanks are also due to Dr. David Ferguson of the Department of Pathology, Edinburgh University who prepared the transmission electron micrographs.

Mr. David Bewsey prepared the colour prints and also assisted with the scanning electron microscopy and Mrs. Marian Sims prepared many of the histological sections. Miss Carol Toolin gave valuable technical assistance with the tissue culture.

To Mrs. Violet Tansey go my grateful thanks for her rapid and expert typing of the manuscript.

Last, but not least, I would like to thank my husband for enduring the last four years with me.

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Clinical Oncology 1979, **5**, 299–303

Cultural characteristics of an apocrine variant of human mammary carcinoma

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(*Accepted for publication 19 July 1979*)

By the use of a tissue culture technique functioning apocrine cells have been demonstrated in mammary carcinoma. The implications are briefly discussed.

Introduction

The presence of cells of apocrine type is a common finding in mammary carcinoma, but its presence is not usually commented on in reports, for supposed lack of clinical significance (McDivitt, Stewart & Berg, 1968). The diagnosis of apocrine carcinoma is rarely made, possibly because of the inability to demonstrate apocrine activity in histological sections of the tumour. Apocrine and holocrine secretion consist of exfoliation of part or the whole of the cell at the free surface of an epithelium. The cells of an invasive malignant neoplasm are often without access to a free surface and so the process may not be observable. In tissue culture the tumour cells are artificially presented with a free surface and abundant exfoliation occurs, as described below. There is reason to believe that the process takes place in vivo and could be expected to influence the facility of invasive growth, and hence of metastasis.

Materials and methods

The tumour described and illustrated was one of a series of 32 breast cancers established in tissue culture over a period of approximately 1 year, primarily to compare the cultural characteristics of highly elastotic, moderately elastotic and non-elastotic tumours. These three categories have been shown to confer corresponding, widely differing prognoses upon patients (Shivas & Douglas, 1972). Fresh tissue was obtained from the Frozen Section laboratory in the Royal Infirmary of Edinburgh and set up in culture.

The procedure used was essentially the 'spillage' technique described by Lasfargues & Ozzello, 1958. The culture medium used was RPMI 1640 supplemented with foetal calf serum (20%), insulin (10 to 20 μg/ml), penicillin (100 I.U./ml) and streptomycin (100 μg/ml). Using aseptic precautions throughout, 2 to 3 mm^3 pieces

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of biopsy material were collected and stored overnight at 4°C in complete culture medium. The tissue was then removed, washed twice with phosphate buffered saline [(P.B.S.)—Dulbecco's formulation without Ca and Mg] and placed in a sterile petri dish containing 10 ml complete medium. It was sliced carefully to release tumour cells from the stroma into the P.B.S. which was then transferred, using a Pasteur pipette, to a test tube where it was allowed to sediment under unit gravity for 30 min. The supernatant above the cell pellet was then discarded. Fresh complete medium was added to the cells and the suspension transferred in 5 ml aliquots to No. 25 Nunclon Tissue culture flasks. The pH was adjusted to 7·3 and the flasks were incubated at 37°C.

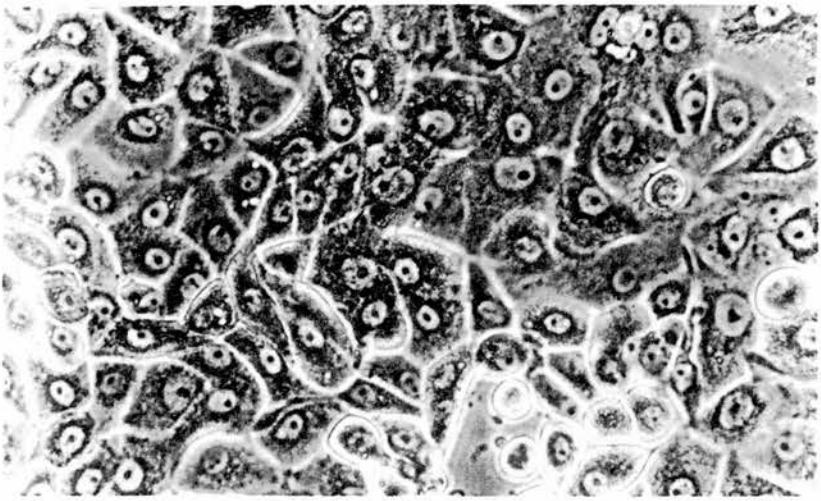

Figure 1. Field from a colony of carcinoma cells growing in tissue culture. The abundant, coarsely granular cytoplasm of apocrine cells is well shown. ×220.

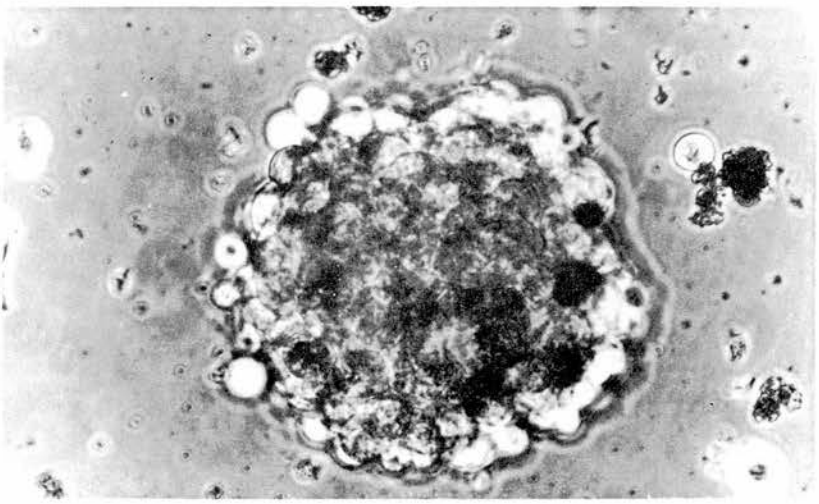

Figure 2. A typical clump of the cells and surrounding debris shed in large numbers into the culture medium. ×220.

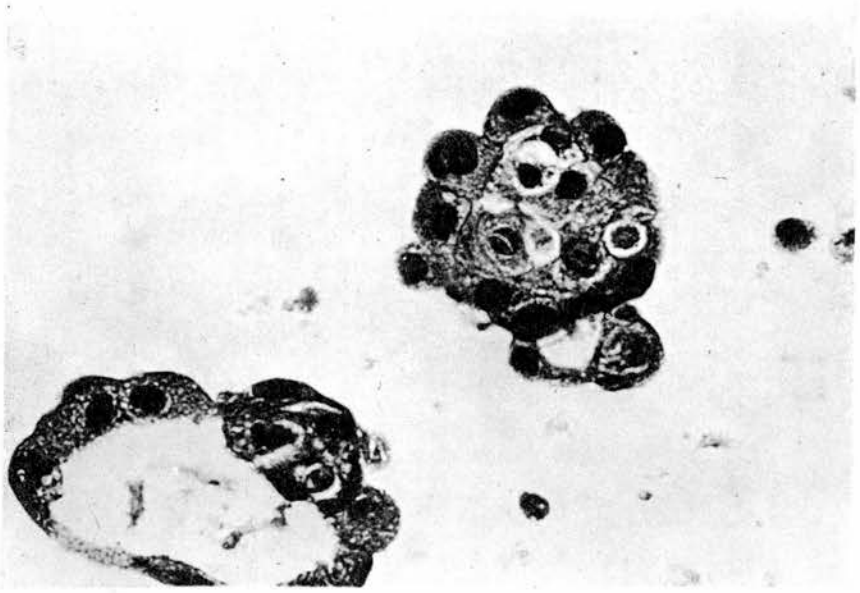

Figure 3. Exfoliated cell clumps (as in Figure 2) in paraffin section, obtained by centrifugation and preparation of a block. The abundant, granular eosinophilic cytoplasm of apocrine cells is again well shown (cf. Figure 1). H & E ×500.

Cultural characteristics

Initially, the culture behaved as others, establishing colonies of epithelial cells on the floor of the flask (Figure 1). However, after approximately 3 days a striking phenomenon was observed in that large quantities of cells and debris were shed into the medium (Figure 2). The nature of this phenomenon was studied histologically. Samples of the medium were centrifuged at 1000 r.p.m. for 5 min and liquid agar used to 'bind' the material. This was followed by formalin fixation, preparation of a paraffin block and of sections stained with Haematoxylin and Eosin. These revealed cytoplasmic fragments, individual cells and small clumps of cells of characteristic apocrine morphology (Figure 3).

Discussion

Review of the biopsy and comparison with sections of the exfoliated debris indicated the presence of a high proportion of cells of apocrine type. Large cells with abundant eosinophilic and granular cytoplasm in the two samples seemed identical (Figures 3, 4 and 5).

The findings support the use of the term apocrine for such cells. Apocrine metaplasia is a frequent component of fibrocystic disease of the breast (mammary dysplasia) though some prefer the term 'pink cell metaplasia' to describe the condition as an indication of doubt concerning its morphological status. Our findings support the view that some carcinomas contain apocrine and holocrine cells, though it may well be that not all such carcinomas arise in areas of apocrine metaplasia. An alternative source for such cells is by metaplasia of the tumour cells themselves.

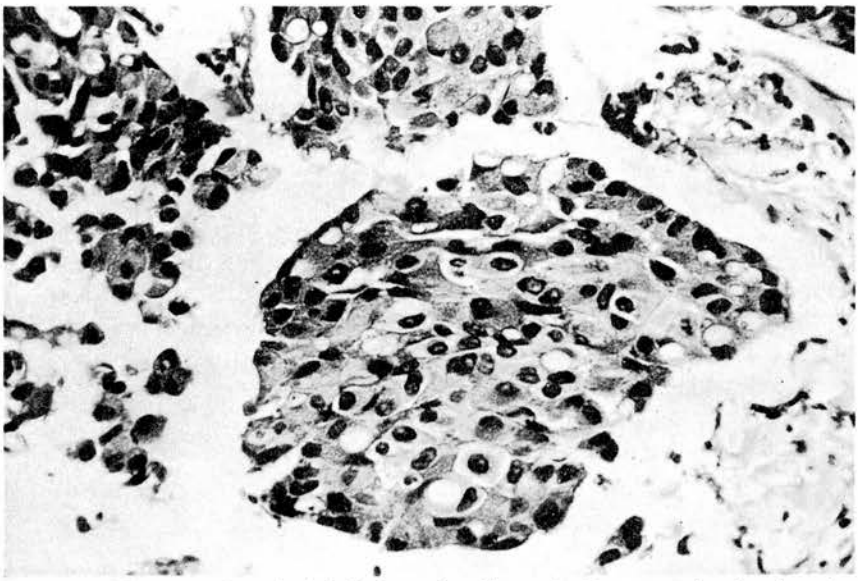

Figure 4. A typical field from the diagnostic biopsy, again showing clearly the apocrine characteristics. H & E ×320.

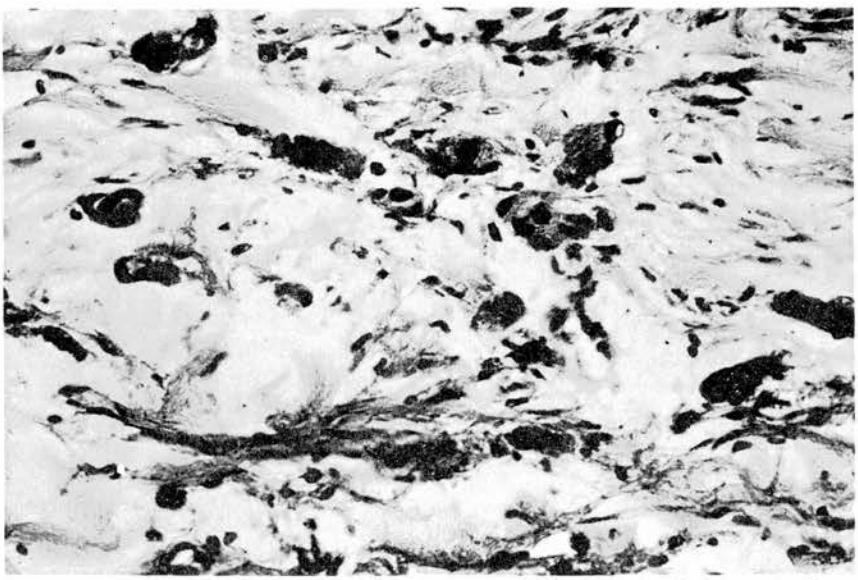

Figure 5. Another field from the biopsy. Extrusion of cell clumps similar to those seen in culture is the conspicuous feature (cf. Figures 2 and 3). H & E ×320.

Diminished mutual adhesiveness of tumour cells is one of the concepts used in explaining invasive growth in malignant tumours, based on changes observed in cell membranes. These *in vitro* findings lend support to its being a feature of human mammary carcinoma. Exfoliated individual cells and small groups of cells (Figures 3, 4 and 5) are present both in the biopsy and in the tissue culture material. The technique could be used for further studies to determine whether a relationship exists between apocrine cell presence and the behaviour of mammary carcinoma, and retrospective studies also appear to be worthwhile.

References

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TABLE II. CELLULAR DISTRIBUTION

NON-MALIGNANT LESIONS AND "NORMAL" TISSUE

No.	Diagnosis	TYPES OF CELLS CULTURED															
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
		'Typical' Epithelial groups	'Elongated' epithelial cells	'Bridged' epithelial cells	'Neat' epithelial cells	Granular epithelial cells	Very pleomorphic cell pavements.	Indistinct cells with cellular projections	'Round' cells	'Elastotic' cells	Giant cells	'Fairy' cells	Cell spheres	'Globules'	Fibroblasts	Myofibroblasts	Unidentifiable cells
5	Fibroadenoma	■	■	■													
8	Fibroadenoma						■								■		
17	Giant fibroadenoma			■	■												
34	Fibroadenoma		■	■	■												
2	Typical fibroadenosis						■	■									
6	Florid fibroadenosis			■											■		
7	Fibroadenosis			■				■							■	■	
11	Fibroadenosis		■	■			■		■						■		
19	Active fibroadenosis. Apocrine component	■	■			■	■	■							■		
45	Fibroadenosis in lactating breast				■				■						■		
4	Fibrocystic disease	■															
9	Simple fibrocystic disease	■	■	■											■		
12	Fibrocystic disease with considerable adenosis and an apocrine component.	■				■	■				■					■	
22	Fibrocystic disease with much stromal collagenisation	■															
70	Gynaecomastia	■	■	■													
35	"Normal" breast	■		■													
37	4½ month pregnant breast		■	■		■											

TABLE IV CELLULAR DISTRIBUTION

HIGHLY ELASTOTIC TUMOURS

No.	Diagnosis	TYPES OF CELLS CULTURED															
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
		'Typical' Epithelial groups	'Elongated' epithelial cells	'Bridged' epithelial cells	'Neat' epitheial cells	Granular epithelial cells	Very pleomorphic cell pavements.	Indistinct cells with cellular projections.	'Round' cells	'Elastotic' cells	Giant cells	'Fairy' cells	Cell spheres	'Globules'	Fibroblasts	Myofibroblasts	Unidentifiable cells
10	Anaplastic carcinoma. Marked elastosis		■												■	■	
27	Anaplastic carcinoma. Marked elastosis	■		■						■							
31	Anaplastic carcinoma. Prominent elastosis. Moderate "stromal reaction"	■		■			■	■		■							
52	Highly elastotic anaplastic carcinoma	■	■					■		■							
62	Anaplastic carcinoma. Mainly large cell Much "stromal reaction". Marked elastosis	■	■	■	■		■								■		■
65	Anaplastic carcinoma, mainly small cell. Grade II elastosis. Apocrine component.	■	■		■	■	■										■
66	Highly elastotic anaplastic carcinoma. Apocrine component.				■					■					■		
67	Lobular carcinoma with a high degree of elastosis.		■		■		■		■	■		■			■		■
68	Anaplastic carcinoma. Grade II elastosis Apocrine component			■	■	■				■			■			■	
69	Highly elastotic anaplastic carcinoma									■							
76	Anaplastic carcinoma. Grade II elastosis Apocrine component					■			■	■					■	■	
80	Anaplastic carcinoma. Grade II elastosis		■				■	■	■	■					■	■	
82	Anaplastic carcinoma. Grade II elastosis Apocrine component	■		■		■				■	■						■
83	Anaplastic carcinoma. Grade II elastosis. Apocrine component	■			■	■	■			■		■		■	■	■	
86	Anaplastic carcinoma. Grade II elastosis. Apocrine component.					■				■							

TABLE III CELLULAR DISTRIBUTION

TUMOURS OF ELASTOTIC INDEX "0" AND "I"

No.	Diagnosis	TYPES OF CELLS CULTURED															
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
		'Typical' Epithelial groups	'Elongated' epithelial cells	'Bridged' epithelial cells	'Neat' epithelial cells	Granular epithelial cells	Very pleomorphic cell pavements.	Indistinct cells with cellular projections.	'Round'cells	'Elastotic' cells	Giant cells	'Fairy' cells	Cell spheres	'Globules'	Fibroblasts	Myofibroblasts	Unidentifiable cells
1	Intraduct carcinoma		■				■								■		
3	Anaplastic carcinoma	■	■	■											■		
16	Lobular carcinoma						■										
20	Scirrhous carcinoma with some elastosis	■													■		
21	Encephaloid carcinoma	■	■	■			■										
23	Anaplastic carcinoma, partly intraduct plus fibroadenosis			■	■	■	■	■	■								
24	Intraduct carcinoma with considerable invasion and an apocrine component				■	■		■		■							
26	Anaplastic, mucoid carcinoma with an apocrine component				■	■							■		■		
28	Anaplastic carcinoma. Little "stromal reaction".				■												
29	Intraduct carcinoma		No growth														
30	Anaplastic carcinoma, moderate elastosis and an apocrine component				■	■				■					■	■	
32	Anaplastic carcinoma, some trabecular architecture. Apocrine cell type												■	■	■		
33	Anaplastic, small cell carcinoma. Moderate "stromal reaction". Moderate elastosis	■		■	■			■	■	■							
36	Anaplastic carcinoma. Moderate "stromal reaction". Apocrine component.		■	■				■					■				
38	♂ encephaloid breast carcinoma. Apocrine cell type				■	■							■				
39	Scirrhous carcinoma				■												
40	Anaplastic carcinoma. Moderate "stromal reaction". Moderate elastosis	■															
41	Anaplastic carcinoma. Mild to moderate "stromal reaction"		■	■				■									
42	Anaplastic carcinoma. Variable "stromal reaction". Moderate elastosis		■	■						■							
46	Anaplastic carcinoma with apocrine cell type					■	■	■					■	■		■	
47	Anaplastic carcinoma. Variable "stromal reaction". Apocrine cell type.					■				■							
48	Mucoid carcinoma with fibrocystic disease and apocrine component.	■			■	■	■	■					■				■
49	Adenocarcinoma with apocrine component					■		■					■				
51	Apocrine variant of mammary carcinoma		■			■				■			■			■	
53	Anaplastic carcinoma. Little "stromal reaction". Some lymphoid infiltration.											■			■		
55	Cystosarcoma phyllodes.	■										■			■		
57	Anaplastic carcinoma with apocrine component.	■		■			■		■			■		■	■		
59	Anaplastic carcinoma. Little "stromal reaction". Some focal necrosis.				■												
61	Scirrhous and medullary carcinoma with prominent lymphoid component.						■					■			■		
63	Medullary carcinoma with scirrhous areas	■							■			■			■		
72	Anaplastic carcinoma. Moderate "stromal reaction". Apocrine component.												■				
73	Anaplastic carcinoma. Slight "stromal reaction". Apocrine component	■	■			■	■			■			■		■		
74	Anaplastic medullary carcinoma, of necrosis and an apocrine component.					■	■					■	■			■	
75	Anaplastic carcinoma. Little "stromal reaction".	■	■	■								■			■		
77	Anaplastic carcinoma. Some elastosis									■					■		
78	Lobular Carcinoma. Some elastosis		■	■			■				■				■		
79	Anaplastic carcinoma. Large cell type. Slight to moderate "stromal reaction".	■		■													
81	Encephaloid carcinoma with apocrine component.	■				■	■						■			■	
84	Anaplastic carcinoma. Large cell type. Apocrine component	■	■	■			■		■				■			■	
85	Anaplastic carcinoma. Large cell type + Fibrocystic disease and apocrine component					■			■	■	■		■		■		