THE
HISTOGENESIS OF
EXPERIMENTAL BLADDER CANCER

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by

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INTRODUCTION

Transitional cell papilloma of the urinary bladder is a delicate papillary lesion with a thin fibro-vascular core covered by transitional epithelium which is identical to that lining the surrounding normal bladder. Increased mitotic activity or invasion of the underlying connective tissues are not present. The term papilloma is, however, also used by some writers to include papillary tumours where the fronds are covered by a hyperplastic epithelium which shows some cytological atypicality and also a slight increase in mitotic activity but no invasion of the underlying connective tissue. While all the widely accepted classifications of neoplasms of the urinary bladder accept the principle of a varying degree of malignancy of transitional cell carcinomas from a low to a high grade, only some contain provision for a benign neoplasm - a papilloma. The remainder call this lesion by various titles including low grade carcinoma and carcinoma grade 0 or grade 1.

Thus, the classification of the bladder carcinoma registry of the American Urological Association[78] grades the tumours into four grades of malignancy according to the method of Broders[19]. Although Dart[34] using the same material makes provision for a benign papilloma ("simple papilloma") Dean and his co-workers[36] on reviewing the material maintained that "since it is impossible by histological methods to differentiate benign papillomas from those that recur and/or metastasize, the former should be classed as carcinomas.....". The classification of Dukes and Masina[42] does make provision for a papilloma because, while they acknowledge that border-line cases are found where morphologic diagnosis is difficult,

they considered that "if the epithelial cells of a papillary tumour closely resemble normal transitional epithelium and are non-invasive in character the tumour should be called a benign papilloma". The classification of Friedman and Ash[56] also includes a papilloma although Ash had previously stated that because he could not predict with certainty the subsequent behaviour from the histological appearance the term "carcinoma, papillary grade 1" be used[8]. Mostofi[103] regards papillomas as being histologically benign lesions but recommends that they should be regarded by the clinician as potentially malignant.

The lack of uniformity in regard to this lesion is further illustrated by reports on incidence. It is reasonable to assume that there should be only minor differences between the statistical analyses of large collections of bladder tumour cases drawn from the general public yet Dunham and her co-workers reported no papillomas among 631 tumours examined histologically but 185 (29%) grade 1 carcinomas[43]. Miller et al[99] in a review of 1432 biopsies of tumours of the lower urinary tract reported that 26 (1.8%) were papillomas and 622 (43%) were low grade carcinomas. The 1384 cases collected at the bladder tumour registry, Armed Forces Institute of Pathology[36] included only 109 (8%) grade 1 carcinomas while Melicow[96] in a study including 1615 epithelial tumours of the bladder showed that 7% were papillomas and 23% were grade 1 carcinomas.

These differences of opinion and the various names applied to this lesion indicate the difficulties that arise when a histologically innocent lesion on occasions recurs; is associated with similar lesions elsewhere in the lower urinary tract at a later time or progresses to behave as, and look

like, a frankly malignant neoplasm. In some instances this may only reflect poor sampling of tissue for examination so that the malignant tissue is not seen by the pathologist[35, 94]. However, this is not true in every case[111].

The use of the term "papilloma" by the pathologist should imply to the clinician that it will behave in an innocent manner yet Ewert and Summons[51] were able to state that they "have been impressed with the inability to determine the prognosis from the pathologic report". The use of the term "carcinoma" for a benign looking lesion is deemed undesirable by the pathologist. The term "atypical papilloma" solves some of these problems in that, while retaining histologic accuracy it conveys a message of caution to the clinician. Uncertain as to whether or not transitional cell carcinomas of the urinary bladder could arise by malignant transformation of a benign papilloma Friedell and his co-workers[54, 55] preferred the label "papillary hyperplasia" in order to avoid the implications of the term "papilloma". They showed that this lesion behaves in a benign fashion but that morphologically there is an indefinite zone between it and carcinoma characterised by varying degrees of dysplasia. As such, papillary hyperplasia was regarded as a pre-malignant lesion rather than a benign neoplasm with the same provisos that accompany pre-malignant lesions elsewhere in the body - that they do not necessarily proceed to frank malignancy.

The confusion surrounding the terminology of this lesion is due to 1. an inability to define accurately the morphological criteria that indicate the possibility of recurrence or malignant behaviour, 2. incomplete knowledge of the histogenesis of bladder neoplasms and 3. a lack of

knowledge regarding the natural history of the untreated papillary lesion of the type under discussion. Further investigations are necessary. The first requires an extensive multi-disciplinary investigation of morphology and related features with a long term follow up correlated with the type of therapy received and a study of the environmental and social factors which may be related to aetiology and/or enhancement of the disease. As can be appreciated, this type of project is probably best done on a national or international scale. The second, the study of histogenesis, can be performed where people enter a carcinogenic situation, such as those industries known to be associated with an increase incidence of bladder cancer, by regular examination and biopsy of the bladder epithelium of such people. However, it is difficult to persuade the worker to undergo regular cystoscopy nor can one omit treatment, or at least, remove the person from the carcinogenic situation when the earliest signs of epithelial alteration make their appearance. In regard to the behaviour of the untreated lesion there are obvious ethical reasons why this cannot be studied in any meaningful number of human subjects. It is felt, therefore, that in respect to the last two considerations the experimental animal model may be of some help.

The first reported production of urinary bladder carcinomas in the experimental animal was by Hueper and Wolfe[69]. This experiment and most subsequent experiments by many different workers in this field were designed primarily to test the carcinogenicity of suspicious compounds or to study the biochemistry of carcinogenesis. While their aim was not to study the morphology of the developing lesions

this had to be done in order to standardise the biochemical findings. Hueper and his co-workers[68] described in some detail the morphology of the established lesions in the dog's urinary bladder on feeding 2-naphthylamine. Bonser and Jull[16] described the histological changes seen in the mouse urinary bladder after the implantation of paraffin wax pellets containing a variety of carcinogens and Roe[115] illustrated the proliferative and neoplastic changes seen in the mouse urinary bladder in response to prolonged irritation. These last two descriptions were based on the changes seen in the presence of a foreign body implanted by surgical means. Although many of the changes recorded in these two studies have also been found in the human bladder[102] (not necessarily under neoplastic or pre-neoplastic conditions) it is thought that the presence of surgical interference and the presence of the foreign body are undesirable for the study of histogenesis. It was decided, then, to examine the experimental models of urinary bladder carcinogenesis in an attempt to find a suitable system for the study of the morphology of bladder neoplasms. The search for this model took cognisance of several factors, not the least of which was the desirability to use the morphologic data evaluated as a baseline for subsequent studies into the behaviour of the tumours. With this in mind the following criteria were considered desirable:

1. The tumours produced should morphologically resemble those found in the human bladder.

2. The model should be reliable. The most satisfactory situation is where all the treated animals develop malignant or pre-malignant lesions. (In biological experiments this can never be completely relied upon but models where the

percentage of positive results is low are of less value for morphologic studies).

3. The investigator should be able to monitor the experiment by direct visualisation or he should be able to predict results on a time or dose of carcinogen basis.

4. The carcinogen should not be generally toxic to the animal used and should cause cancer only in the lower urinary tract.

5. A relatively short time for tumour development is preferable.

6. The method of administration of carcinogen should be easy and near physiological. Foreign bodies in the bladder should be avoided.

The bladder tumours produced in dogs by feeding 2-naphthylamine are morphologically similar to those found in the human bladder. Cystoscopy of bitches is relatively easy so direct visualisation can be used as a monitoring system. Similar bladder tumours can also be induced in dogs by 4-aminobiphenyl[38, 133] and 4-nitrobiphenyl[39]. None of these compounds produces any measurable toxity and none produces tumours elsewhere in the body. The main disadvantage to the use of the dog as an experimental animal are 1. the size of the animal and what that means in housing facilities and the large amounts of carcinogen consumed - factors which are exaggerated by the use of a large number of animals and 2. the long induction periods required for tumourigenesis (104 weeks for 2-naphthylamine[68], 143 weeks[133] and 128 weeks[38] for 4-aminobiphenyl and 130 weeks for 4-nitrobiphenyl[39]) which also exaggerates the first advantage.

Of the smaller animals the mouse and the rat are those most commonly used in bladder cancer studies and numerous

compounds have been shown to be carcinogenic for the urinary bladders of these animals. Clayson and Cooper[25] give a comprehensive list of these compounds but most produce tumours in only a small percentage of the animals tested and are, therefore, not considered here. Two compounds, when tested by the bladder implantation technique[4,73], give a higher yield of tumours. Thus Clayson et al[26] produced bladder tumours in 98% of 44 mice when 2.4.xylylazo-2-naphthol (oil orange KB) was incorporated into wax pellets and implanted into mouse bladders and Bonser et al[15] produced carcinomas in 58% of mice using 20-methylcholanthrene in a similar system. As pointed out above, however, the presence of a foreign body, surgically implanted into the bladder, is considered undesirable for morphologic studies.

Among the materials given systemically , 4-ethyl-sulphonyl-naphthalene-1-suphonamide has produced 18 carcinomas in 39 female mice fed the compound but only 5 carcinomas in 34 male mice similarly treated[23]. Dibutylnitrosamine, when fed by mouth to rats produces carcinomas in many organs including the urinary bladder[87]. This multifocal action is undesirable but Druckrey et al[41] reported that the subcutaneous injection of this compound induced bladder cancers in all of 14 rats tested and produced only three extra-vesical carcinomas. He further states that 4-hydroxybutyl-butylnitrosamine, when given in the drinking water produces only bladder cancers and those within 280 days. This appeared to be a promising model except that the authors describe the tumours as squamous carcinomata. While the term "squamous" is not clarified by illustration, taken at face value this would be unlike the majority of human bladder cancers. However, since starting the experimental work described in this present report Ito

and his co-workers [71] have described the morphology of the lesions produced in the rat urinary bladder at varying stages when N-butyl-N-(4-hydroxybutyl)nitrosamine was added to the drinking water. They showed that the tumours produced were transitional cell tumours and their report indicates that this model fulfils most of the criteria enumerated above.

In 1967 Ertürk et al[50] described the carcinogenicity of N-(4-(5-nitro-2-furyl)-2-thiazolyl)formamide to the urinary bladder of rats when fed by mouth. Later reports[49,126] have confirmed that all rats fed this compound have developed malignant or pre-malignant lesions of the lower urinary tract, including papillary lesions, but no tumours outside this system. As this model fulfilled other criteria listed above - invasive carcinomas were produced within 22 weeks with little evidence of toxicity - it was chosen for further study. It has been subsequently shown that the investigator is able to predict results on a time basis[124b].

The purpose of this presentation is to describe the histogenesis of experimental bladder cancer because it is believed that there is inadequate documentation of the manner in which these lesions develop. It will be shown that, in the model chosen, bladder neoplasms develop along one of two lines - an exophytic or papillary line and an endophytic line. It will also be shown that the latter, the endophytic type, is a less well differentiated lesion with a greater mitotic activity than the exophytic lesion and that the endophytic lesion becomes an invasive carcinoma at an earlier stage than the exophytic type. This is primarily a light microscopic study. Electronmicroscopy has been used on a limited scale only, mainly to highlight morphologic differences between the exophytic and endophytic growth patterns. Autoradiography,

using tritiated thymidine, has been used to label cells in the synthetic phase of the mitotic cycle in an attempt to identify the proliferating cells and second to assess the mitotic activity. My purpose has been to present the pertinent facts as I see them. Isolated, apparently irrelevant events have, largely, been omitted. I ask that this omission be forgiven in order that the whole may be seen more clearly.

MATERIALS AND METHODS

The findings presented in the results section of this report are the product of a single investigation and the materials and methods described in this section are those of that investigation which incorporated light microscopy, autoradiography and electron microscopy. Where results from other experiments of the author have been used to illustrate a point in the discussion the methodology as concerns strain and sex of animals, concentration and type of carcinogen and general experimental procedure is the same unless specified.

One hundred and five caesarian delivered, male Fischer rats (Charles River Breeding Laboratories, Massachusetts) being approximately 5 weeks old and having a mean weight of 67 grams, were labelled by ear punching and housed six to a cage in a room with a constant air temperature of 72°F. These animals, which will be subsequently referred to as "test" animals, were fed a diet of powdered laboratory feed (Lab Chow, Purina) with added N-(4-(5-nitro-2-furyl) -2-thiazolyl)formamide (FANTF), 0.188% by weight. (Saber Laboratories, Morton Grove Illinois). This compound was first ground with an equal weight of granulated sugar and then mixed into the powdered food using a mechanical mixer for 30 minutes. Batches of stock diet were mixed approximately once a week and stored at room temperature. At the same time, 58 similar animals, with a mean weight of 72 grams, to act as controls, were kept under identical conditions but fed unaltered powdered lab chow. Both groups were given food and water ad libitum. All animals were examined and weighed at weekly intervals. All food consumption was

recorded per cage and the weekly consumption of carcinogen was calculated as a mean for each animal in each cage.

From the end of the third week, after the commencement of feeding, through the 20th week, groups of from 3 to 6 test animals were sacrificed weekly (table 1) by the intra peritoneal injection of sodium pentobarbital. Groups of control animals were sacrificed at appropriate intervals (table 1) by the same method. Thereafter, groups of both test and control animals were sacrificed at the ends of the 22nd., 25th., 30th. and 35th. weeks. Full autopsies were performed on all animals. Sections of liver and both kidneys were taken for histological examination from every animal. Mammary gland tissue, small bowel and both adrenal glands were examined macroscopically in every case but histologically in approximately one third of them. The urinary bladders to be used for light microscopy were removed from the body after clamping the urethra below the prostate gland and freeing the bladder, with the prostate, distal to the clamp. The bladder, with the clamp still attached was then transferred to a dish containing Bouin's fixative. If urine was present within the bladder this was first removed by syringe and fine needle inserted through the posterior wall of the bladder midway between the fundus and the trigone. The bladder was then filled via the same syringe and needle with Bouin's fluid, using a three way stopcock which allowed the emptying and filling to be performed without disturbing the needle inserted into the bladder. Fixative was injected until all creases were removed from the bladder wall but great care was taken not to distend more than this. After approximately one minute, fixation was sufficient so that,

when the needle was removed, there was no elastic recoil of the bladder wall. The bladders were then transferred to separate, labelled containers of Bouin's fluid and kept for 2 hours whereupon they were bisected in the mid-sagittal plane. The external surface and the mucosa were examined with a hand lens (X10), the prostate was trimmed away, and the bladder halves were transferred to 75% ethanol. Other tissues taken for histology were fixed in Bouin's fluid for 2 hours, trimmed, replaced in Bouin's fluid for a further 2 hours and then transferred to 75% ethanol. Thereafter, all tissues were dehydrated with graded alcohols in preparation for paraffin embedding.

Sections of all tissues were routinely stained with haematoxylin and eosin (H&E). As required the following stains were also performed: periodic acid Schiff (P.A.S.), with and without previous digestion with diastase; alcian blue; Giemsa stain; Verhoeff elastic stain with a van Gieson counter stain and silver impregnation for reticulin fibres.

For the study of deoxyribonucleic acid (DNA) synthesis, tritiated thymidine (H^3-thymidine) was injected subcutaneously, one hour before sacrifice, into groups if six test and six control animals at pre-selected dates (table 1). In all instances, injections were given at 08.00 hours and the animals were sacrificed at 09.00 hours. H^3-thymidine was supplied by Swartz Bioresearch Inc. and had a specific activity of between 10.6 and 11.5 C/m.mole. and contained 0.5mc/ml in sterile water of pH 6.5. It was injected in a dose of 0.1mc/100gms body weight (0.2ml/100gms body weight). The tissues from these animals were treated in an identical fashion to that described above but in addition autoradiographs were prepared in the following manner. Duplicate slides were

prestained with H&E, dried and then dipped in undiluted Kodak Nuclear Track Emulsion - NTB3 - heated to 40°C. One set was left to expose for 14 days and the other for 28 days at 4°C with calcium chloride as a desiccant. The radiographs were developed using Kodak D19B developer, diluted 1 in 4 with water, for 4 minutes at 70°C and fixed in half strength fixative. The sections were mounted in canada balsam.

Assessments of mitotic activity were expressed as a labelling index which was calculated as the percentage of labelled nuclei in any lesion examined. Both the total number of nuclei and the number of labelled nuclei were counted and in order to avoid errors of selection all the nuclei in any one lesion being assessed were included.

For the electron microscopic studies, groups of animals were sacrificed at preselected intervals as shown in table 1. The abdomen was first opened under sodium pentobarbital anaesthesia. Urine, if present, was removed from the bladder via a fine needle inserted through the posterior wall. The bladder was then filled, in situ, with a solution of 5% gluteraldehyde in a 0.2M sodium cacodylate buffer, pH 7.4. At the same time the identical fixative was dropped on to the outer surface of the bladder. After approximately one minute the bladder was removed from the body and transferred to a small dish containing the same fixative where it was opened, examined with a hand lens and areas selected for electron microscopy. These small samples were cut free with a sharp blade and trimmed into thin strips of tissue and placed in 5% gluteraldehyde in sodium cacodylate buffer, pH 7.4, for between 2 and 4 hours at room temperature. Thereafter, the fixative was decanted and the tissue left in

sodium cacodylate buffer, pH 7.4, overnight in the refrigerator. The tissue was then post fixed in 2% osmium tetroxide in Millonig's phosphate buffer[100] for 2 hours, washed three times with maleate buffer, pH 5.2, and then placed in 2% uranyl acetate solution in maleate buffer for one and a half hours, again washed in maleate buffer, dehydrated with graded alcohols and infiltrated and embedded in Araldite in flat moulds.

Comments:

There are several points in the foregoing account of the materials and methods used that require further explanation. They are better discussed at this stage rather than in the general discussion.

The use of weanling rats was empirical. Male rats were chosen in preference to female animals for several reasons. It was planned to use this model, if suitable, for studying the kinetics of developing bladder tumours and also for the study of exfoliative cytology. It was not known what effect the normal oestrus cycle would have on these parameters. Further, some of the tissues of the lower urinary tract are oestrogen sensitive with a tendency to squamous metaplasia[6,70,138]. This was regarded as an undesirable possibility which may have affected female rats.

The concentration of 0.188% N-(4-(5-nitro-2-furyl)-2-thiazolyl)formamide was taken from the work of Ertürk et al[50]. This concentration was chosen by them as being equimolar to 0.2% formic acid -2-(4-(5-nitro-2-furyl)-2-thiazolyl hydrazide[20] one of several related carcinogens being studied by the Division of Oncology, University of Wisconsin Medical School[101]. The carcinogen was first mixed with granulated

sugar because, due to the water content of the compound, it was sticky and would not mix evenly with the powdered feed in the mechanical mixer. It was found that when it was ground together with sugar the resultant mixture diffused easily and evenly through the diet. No alternate method of administering the carcinogen was considered when it was shown that the compound was not water soluble.

There were several problems concerning the preparation and handling of the carcinogenic diet. Of these the most important was the possible danger to those handling the compound and to those working in the same vicinity. A special "kitchen" was provided, adequately screened from all other laboratory facilities and used only for the purpose of mixing the diet. An extraction fan was installed which was used only when the room was not occupied. Before use the fan was switched off to allow any particles in the air to settle. Whenever the carcinogen was handled the investigator was gowned and wore disposable gloves and mask. Within the animal room sawdust, as an absorbing agent for excreta, was replaced by large sheets of soft cardboard which reduced the risk of aerial contamination.

The urinary bladder of the rat can be inflated via the urethra either in situ or after removal from the body. The reasons for using a per urethral technique is to avoid damaging small lesions on the bladder mucosa when one is performing tests for carcinogenicity. Preliminary studies using FANTF showed that all animals developed tumours or epithelial alterations and that most of these were on the anterior wall. Further, the protocol called for a mid sagittal section. Therefore, because technically it was easier, the bladders were inflated through the posterior wall.

The animals receiving H^3-thymidine were treated at the same time on each occasion so that comparisons could be made between one stage and another. Fixing the time removed the variable introduced by the circadian rhythm of mitotic activity[80,127]. The photography of autoradiographs presents a difficulty in that the exposed grains of emulsion lie at a different level to the tissue. This is partly reduced by thinning the emulsion by heat or dilution. The photographic difficulties are worst when using the highest power objectives. In this report the illustrations of autoradiographs have been focused to give the best compromise result.

The tissue used for electron microscopy was trimmed into strips, rather than cubed, because it was found that in this manner primary orientation of the tissue in the moulds was facilitated. Flat moulds were used for the same reason.

TABLE I

Weeks			
3		HT6;HC6.	
4	T3;C2.		ET3;EC1.
5	T6.		
6	T5.		
7		HT6;HC6.	
8	T3;C2.		ET3;EC1.
9	T6.		
10	T5.		
11		HT6;HC6.	
12	T2;C2.		ET4;EC2.
13	T5.		
14	T3.		
15		HT6;HC6.	
16	T3;C2.		ET3;EC1.
17	T3.		
18	T3.		
19	T3;C3.		
20	T2.		
22		HT6;HC6.	
25	T2;C2.		ET2;EC1.
30	C3.	HT3.	
35	C6.	HT3.	

T - Test animals, C - Control animals.
H - animals given H^3-thymidine for autoradiography.
E - animals used for electron microscopy.

RESULTS

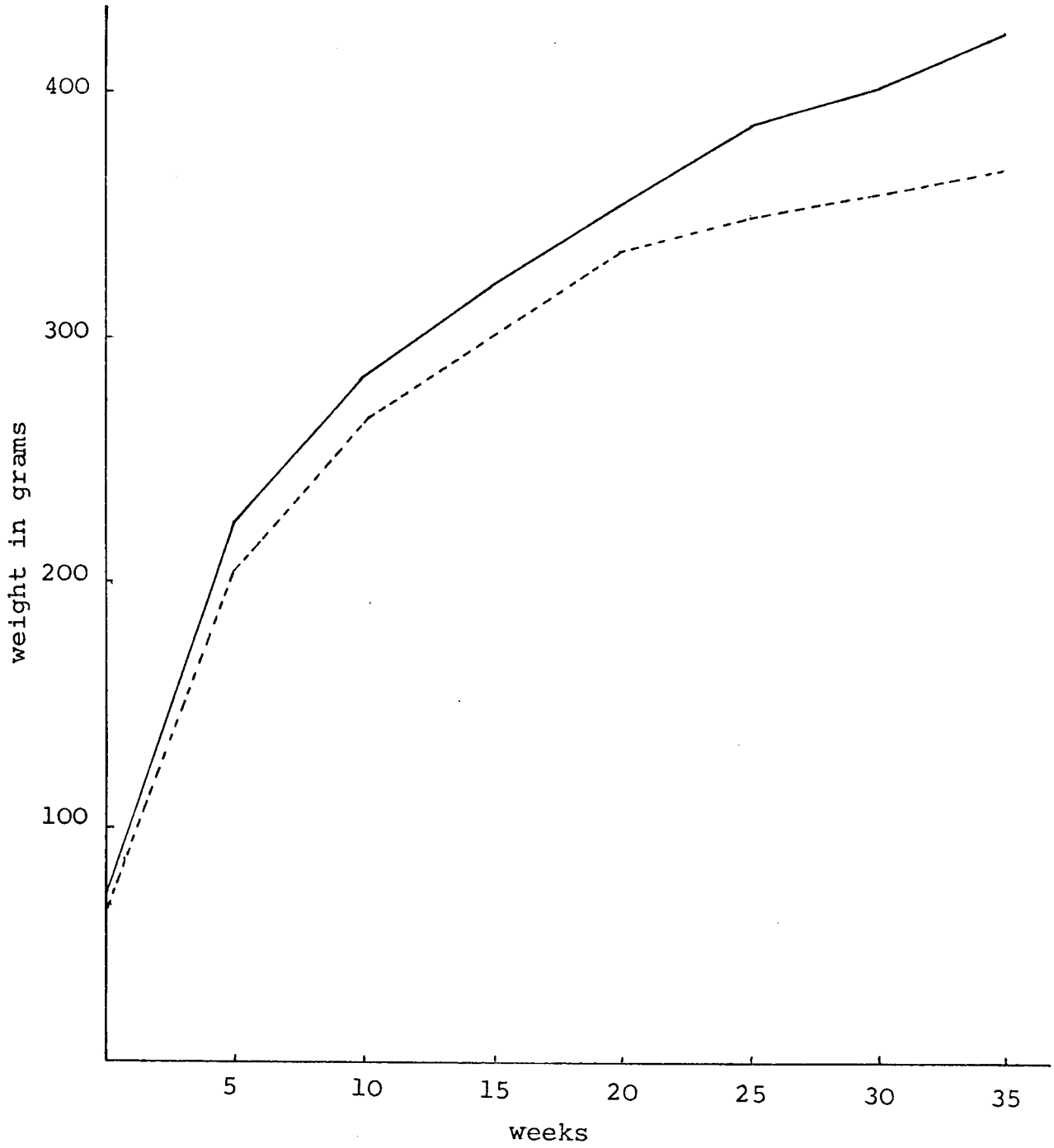
For the ease of description the results of this experiment are described in four separate sections: 1. the general experimental results other than the morphology of the urinary bladder; 2. the morphology of the normal rat urinary bladder as seen in the control animals; 3. the morphology of the urinary bladders of rats fed carcinogen and 4. autoradiographic data concerning the degree (as opposed to locality) of mitotic activity.

1. General.

Autopsies revealed no abnormality in any control animal. All animals were free of the bladder nematode Trichosomoides crassicuda. In the test animals abnormalities were restricted to the urinary bladder except in two instances. In one animal, sacrificed at week 30, the epithelium covering one renal papilla was hyperplastic. In another animal, sacrificed at week 35, there was a secondary carcinoma metastasis in the left groin.

The mean amount of carcinogen consumed per animal was 0.193 gms per week (range - 0.150 gms at week 3 to 0.220 gms at week 25). That the carcinogen might have a slight toxic effect is shown by the differences in weight gain between the test and control animals (text fig. 1). The difference between the two curves is statistically significant ($t = 56$; $p < 0.01$). While none of the test animals ever appeared distressed macroscopic haematuria was noted during the last few weeks of the experiment.

Text Figure 1.



Weight gain for test animals (broken line) and control animals (solid line) against duration of feeding in weeks. Plotted in 5 weekly intervals for convenience.

2. The Normal Rat Urinary Bladder.

In the distended state used in this experiment the urinary bladder of the rat is lined by three layers of epithelial cells, although in some areas only two can be clearly discerned (fig. 1). The most superficial layer is composed of cells which are larger than those underneath so that each superficial cell covers several smaller underlying cells. These superficial cells have larger nuclei than the deeper cells and are occasionally bi-nucleate. The cytoplasm of the superficial cells is generally paler staining than that of the underlying epithelial cells when examined in H&E stained sections. The cells of the intermediate and basal layers show no particular features on light microscopy. The sub-epithelial connective tissue is composed of collagen and loose fibrous tissue within which run the blood vessels. Capillaries reach up from sub-epithelial arterioles to bulge into the undersurface of the epithelium (fig. 2). Interlacing muscle bundles are situated in the outer half of the bladder wall and are covered by a thin serosa. There is no muscle immediately beneath the epithelium lining the bladder lumen and there is no demonstrable elastic tissue in the bladder wall.

Autoradiographs show that DNA synthesis is uncommon but when present is seen in cells of both the deep and superficial layers but more often in the basal layer (fig. 3). Rarely, cells within the sub-epithelial connective tissues were labelled. (fig. 4). Labelled endothelial cells were not observed.

The ultra-structure of transitional epithelium shows several characteristic features (fig. 5). The superficial cells are bounded on their luminal aspect by a double membrane. This membrane has a thicker luminal component and a thinner

component on the cytoplasmic aspect and is referred to as the asymmetric unit membrane (AUM). The surface of the cell presents a very angular appearance due to the presence of angular ridges. Extending down from the surface are clefts lined by AUM. Within the cytoplasm of the superficial cells there are fusiform vesicles, which in the plane of section are not connected to the surface, which are also lined by AUM with the thicker component on the inner, luminal, surface. Another characteristic feature of these superficial cells is the presence of prominent dark bodies which contain negatively stained hexagonal sub-units (fig. 14) and which are referred to as telolysosomes[37]. Fine tonofilaments can be seen in the superficial portion of the cell running roughly parallel to the surface. Adjacent superficial cells are joined at the luminal surface by tri-partite junctions. Immediately beneath this straight tri-partite junction the cell interface becomes interdigitate. The intermediate cells contain occasional telolysosomes and a few vesicles lined by AUM. These structures are consistently absent from the basal cells. The cell boundaries between adjacent intermediate cells, adjacent basal cells and between the cells of the different layers interdigitate in the distended state used in these experiments. Occasionally the intercellular space is slightly widened so that adjacent cells are separated from each other. Desmosomes are seen between cells but they are uncommon. Below the basal layer there is a basal lamina which follows the contour of the basal cells. This basal lamina bridges the gap between adjacent basal cells but in this region it appears blurred. The underlying connective tissue contains collagen, blood vessels and supporting cells. None of these shows any particular features.

There is no change in the morphology of the rat urinary bladder with increased age but there is a reduction in the number of autoradiographically labelled cells in older rats indicating reduced mitotic activity.

3. The Urinary Bladder of Rats Fed Carcinogen.

For the convenience of description the changes noted in these animals may be divided into three stages: a stage of generalised hyperplasia; a stage of localised increased hyperplasia and a stage of tumour development. This division into stages is artificial in that there is a steady progression of changes so that each stage merges with the next. The division is made on light microscopic or macroscopic appearances. Electron microscopy showed no constant nor specific changes. Some ultra-structural disparities between the different lesions to be described were noted and will be reported.

Stage 1 is the stage of generalised reaction in both the epithelium and the sub-epithelial connective tissue. The earliest changes, noted at week 3, were mainly vascular. Thus, there was capillary congestion in the immediate sub-epithelial region and this was associated with a prominence of the arterioles situated a little deeper in the bladder wall (figs. 6 & 7). Evidence of vascular proliferation was found in the presence of labelled endothelial cells in the autoradiographs (fig. 8). In addition, while there was no measurable increase in the amount of connective tissue there was an increase in the number of labelled connective tissue cells seen in the autoradiographs.

The early epithelial changes, noted at this time, consisted of no more than a change in nuclear appearance manifest by increased nuclear size and chromatin clumping (fig. 9). The epithelium appeared slightly thicker due to an increase in cell size. Autoradiographs made from animals sacrificed at week 3 showed an increased mitotic activity which was almost entirely confined to the basal layers (Fig. 8). While a few labelled cells could be found in the intermediate layer no superficial cells were labelled. By week 4 the epithelium showed a generalised hyperplasia. In the early stages (week 4 to 7) this hyperplastic epithelium showed some loss of the morphologic variation from the basal to the superficial levels but many of the surface cells still appeared larger with paler staining cytoplasm. By week 8, however, in localised areas only, the whole thickness of the epithelium was replaced by nests of rather similar cells (figs. 10 & 11). These cells showed darker staining cytoplasm than the surrounding cells. In other areas smaller groups of these cells could be found adjacent to the basal area only (fig.12). Autoradiographs made from animals sacrificed at week 7 showed that the bulk of the epithelial mitotic activity was present in the basal layers with no labelled cells being found in the superficial cells (fig. 13).

Among the early changes noted electron microscopically was a flattening of the surface cells, many of which contained an increased number of telolysosomes (fig. 14). In other areas of the hyperplastic epithelium the surface cells were separated from each other and cells resembling the intermediate cells were seen bulging through to the surface (figs. 15 & 16). These latter cells contained a paucity of fusiform vesicles in the cytoplasm but were bounded on the

surface by an asymmetric double membrane. In some areas where the full thickness of the hyperplastic epithelium was composed of cells showing no variations from base to surface, the electron microscopic appearance reflected this situation (fig. 17). The cells composing the surface in these areas were covered by an asymmetric double membrane but showed in most instances very few fusiform vesicles lined by AUM in the superficial cytoplasm. The surface of these cells, instead of having angular ridges, showed rounded humps. The cytoplasm of these cells did not show the relative electron translucency as seen in the surface cells of the normal bladder.

Stage 2 is a transition stage between the generalised hyperplasia and the development of localised tumours. In this stage two forms of lesion were observed - an exophytic and an endophytic form.

The exophytic pattern was characterised by outfoldings, into the bladder lumen, of the full thickness of the hyperplastic bladder mucosa to form micropapillae (figs. 18 & 19). These had a thin core of connective tissue carrying a capillary and were covered by epithelium similar to that lining the surrounding bladder. In most of these micropapillae there was a variation of cell type from the basal to the surface layers, similar to that in the normal bladder, so that the surface cells appeared larger with pale staining cytoplasm. In some instances many such micropapillae were seen grouped close together.

The endophytic growth was manifest by the bulging down of the hyperplastic epithelium into the underlying bladder wall (figs. 20 & 21). The inferior aspect of this growth

pattern presented rounded broad fronts of epithelium to the partially compressed connective tissue producing a nodular appearance. These nodules were delineated by this underlying connective tissue and by capillaries which reached up between the nodules to within three cell layers of the luminal surface. The epithelial cells composing the nodules showed little morphologic variation from the basal layer to the surface but appeared rather similar to each other. They were moderately large with eosinophilic staining cytoplasm and central round nucleus containing an unobtrusive nucleolus and showing minimal chromatin clumping. At the surface some larger cells could be found which were similar to those in the normal bladder and these were stretched out over the underlying hyperplastic epithelium. This lesion was labelled "nodular hyperplasia".

Localised lesions were also seen which showed the presence of both the exophytic and endophytic growth patterns (figs. 22, 23, 24 & 25). These complex "combined" lesions took many forms, no two being alike, and appeared as multiple in and out foldings of the hyperplastic epithelium.

Differences between the papillary and the nodular lesions could also be found on electron microscopy. The surface cells of the papillary lesions appeared similar to those in the normal bladder with an angulated surface composed of AUM and many fusiform vesicles lined by AUM in the cytoplasm. On the other hand, many surface cells of the nodular lesions had few fusiform vesicles and the surface membrane was not angulated but presented with rounded undulations (fig. 26). In both instances the membrane itself appeared similar to that seen in the normal bladder. Telolysosomes were present in small

numbers in both the superficial and the immediate underlying cells of the papillary lesions but were rarely found in the nodular lesions. The cells of the papillary lesions were always closely applied to each other. These cell boundaries were often straight with loss of the complex interdigitation which was only seen at the junction of three or more cells (fig. 27). The intercellular junction of the nodular lesions did show interdigitation. In most of the nodular lesions there was a widening of the intercellular space so that adjacent cells were separated from each other (fig. 28). Within this space were interdigitating projections of the cells (fig. 29). Desmosomes were not generally present in these areas.

In all these lesions the mitotic activity, as shown by autoradiography, was confined to the deeper layers of the epithelium (fig. 30). When telophases were found in micropapillae the direction of separation indicated a division parallel to the basement membrane (fig. 31). In the nodular lesions the telophases were orientated in a haphazard way showing no particular direction of division (fig. 32). A few labelled cells could be found in the thin connective tissue core of the micropapillae but it could not be ascertained whether or not these were labelled endothelial cells (fig. 33).

Stage 3 is the tumour stage. As used here a tumour is defined as a localised growth which can be seen macroscopically. As such there is an indefinite boundary between this and the preceding stage. The bladders of all test animals surviving for longer than fourteen weeks showed the presence of at least one tumour. Fifty per cent of the animals sacrificed

between week 8 and week 14 showed the presence of tumours. The vast majority of these tumours were situated on the anterior wall of the bladder near the fundus. All protruded into the bladder lumen.

Microscopically two types of tumour were recognised, corresponding to the two types of localised hyperplasia described in stage 2. Combinations of the two were also present. The first was a complex papillary tumour, with a thin fibro-vascular core, covered by 5 to 7 layers of epithelial cells showing a morphologic variation from the basal to the surface layers similar to that seen in the hyperplastic epithelium of early stage 1 (figs. 34 & 35). The complexity of the lesion was produced by branching and by infoldings which, when cut transversely, produced a pseudo-acinar appearance. The base of these papillary tumours ranged from narrow to moderately broad (fig. 36). The delicacy of the lesion seemed to depend on the width of the base - the more delicate tumours having a narrower base.

The second type of tumour had a broad base of vascular, loose connective tissue which protruded into the bladder lumen, in a polypoid fashion forming the connective tissue core of the tumour. This core was capped by hyperplastic epithelium having the same configuration as, but more exaggerated than, that seen in nodular hyperplasia (fig. 37). Variation of the morphology of epithelial cells from the basal to the surface layers, although present in areas, was more often absent. The cells were similar to those seen in stage 2 lesions but in some tumours they had a slightly basophilic cytoplasm and a nucleus which contained an obvious nucleolus. These latter cells were often separated from each other by a widened inter-cellular space and had an angular shape. While neither

keratin nor intercellular bridges could be discerned, these areas had a squamoid appearance.

The polypoid lesions were more commonly seen than the papillary lesions. Tumours showing mixtures of the two were found in roughly the same numbers as the polypoid tumours. These combined tumours often showed the polypoid configuration of the second type covered by an epithelium showing the pseudo-acinar arrangement and base to surface variation of the first. Also found were polypoid lesions which showed mainly the nodular type of hyperplastic epithelium associated with papillary formations, usually at the periphery (figs. 38 & 39).

Autoradiographs of these early tumours again showed that the mitotic activity was concentrated in the basal cells (fig. 40). In contrast to the earlier stages, however, an occasional superficial cell was labelled (fig. 41). In the papillary lesions labelled cells could be found in the thin connective tissue core but again it was not possible to identify whether or not these were endothelial cells.

Before week 14 the largest tumour measured 0.2 cm in diameter but after week 14 they became progressively larger and in some instances virtually filled the bladder by week 35. In the closing stages of the experiment all the bladders of test animals showed the presence of several tumours. Usually one, sometimes two, of the tumours in any one bladder was very much larger than the remainder which, while being smaller, ranged in size from being barely perceptible to the naked eye to being fairly large lesions (figs. 42 & 43). Before about the twentieth week the two different growth patterns described above could be recognised in most tumours, either alone or in combination. After the twentieth week, however, as the tumours got progressively larger, the papillary and/or

polypoid structure became more disorganised.

Although infrequent examples of easily recognisable papillary and polypoid lesions could be found as late as week 35 (fig. 44) the disorganisation of structure referred to resulted in different morphologic patterns becoming evident after week 20. In some tumours a very characteristic filigree pattern was first encountered at week 20 and became more conspicuous by week 25. This pattern was characterised by narrow bands of epithelial cells extending down from the surface into the connective tissue core of the tumour but not beyond into the bladder wall (fig. 45). In the early stages (i.e. week 20) these bands were up to 7 cells broad and showed a cellular variation from the periphery to the centre similar to the base to surface variation described in papillary lesions in stage 2 (figs. 46 & 47). With increased duration of the experiment these bands were seen to be narrower and at times were only two cells thick (figs. 48 & 49). At this later stage they were anastomosing. Reticulin stains revealed that in many instances the centres of these bands were lumenated and continuous with the bladder lumen (fig. 50). The connective tissue element in these areas was loose and oedematous. Alcian blue stains revealed only minimal acid mucopolysaccharides . Mitotic activity was present in both the epithelial and connective tissue cells but predominantly in the former. Autoradiographs showed frequent labelling of endothelial cells (fig. 51).

A second, numerically more common, tumour type consisted almost entirely of epithelial cells with very little connective tissue stroma. In some instances these cells were in small clusters, each surrounded by a thin rim of connective

tissue (fig. 52). Elsewhere larger sheets of epithelial cells were present. Now and then acinar structures were seen in these tumours and these showed a configuration similar to that found in the papillary lesions earlier in the experiment (fig. 53). Many of the cells composing these tumours were similar to the cells seen in earlier lesions having a moderate amount of eosinophilic staining cytoplasm and a central round to oval nucleus which showed some peripheral clumping of the chromatin and a small basophilic nucleolus. Another cell type found had a scanty cytoplasm and an increased nuclear/cytoplasmic ratio. A third cell type - a squamous cell - was also seen and is described in more detail later under a discussion of squamous metaplasia. Autoradiographs of these "solid" epithelial tumours showed numerous labelled cells which were scattered throughout the tumour and not confined to the basal layer (fig. 54).

As stated above there were no specific electron microscopic changes characteristic of neoplastic cells. Some showed an increase in smooth endoplasmic reticulin (fig. 55A), occasional cells showed the presence of laminated phospholipid bodies in moderate numbers (fig. 55B), autophagic vacuoles were commonly seen and the basal cells often showed a prominence of tonofibrils as described below in the discussion of squamous metaplasia.

Invasion, or encroachment, by the epithelial element, of the connective tissues of the tumours themselves or of the underlying bladder wall occurred in several ways.

i. The nodular hyperplastic lesions of stage 2 showed expansile growth into the underlying tissue on a "broad front". This caused compression of the collagen stroma which was best shown in P.A.S. stained sections, so that the fibres were

stretched around the advancing edge of the nodules. This form of broad front encroachment was also seen in the polypoid nodular lesions.

ii. A second form of expansile encroachment was found in papillary tumours after week 15. This was characterised by the presence of islands of cells, completely surrounded by collagen, lying apparently isolated in the base of the tumour (fig. 56). The surrounding connective tissue showed compression in a similar manner to that described above. In all instances there was a variation of cell type from the periphery (basal layer) to the centre which was the same as that found in the remainder of the papillary tumour. Sometimes the centre of these islands was lumenated producing a pseudo-acinar appearance. In many instances serial section revealed that these apparently isolated islands represented downgrowths or infoldings of the papillary tumour cut transversely.

iii. The third pattern of invasion was only seen in polypoid nodular tumours from week 14 onwards. This was characterised by the presence of sharp spurs of epithelial cells infiltrating between, rather than compressing, the connective tissue fibres. These sharp spurs of cells were found lying between the nodules of hyperplastic epithelium (fig. 57).

iv. After week 18 small irregular islands of epithelial cells could be found lying in the connective tissue of the bases of both papillary and nodular tumours. The cells composing these islands had little cytoplasm and an increased nuclear/cytoplasmic ratio. There was no variation in cell type from the periphery to the centre as seen in ii above (fig. 58). The connective tissue within which these islands lay was in most instances loose and oedematous. Possibly due

to this there was no compression effect as seen in i. and ii. above and the periphery of these islands was slightly irregular. However, when these islands abutted on dense collagen there was a slight compression effect and the periphery of the cell cluster appeared smooth. Serial sections revealed in some instances that these apparently isolated islands were the result of downgrowths of narrow bands of cells cut transversely.

v. The downgrowths seen in the filigree pattern of tumour growth were similar to those described in iv. These narrow bands of epithelial cells only reached as far as the base of the tumours, where the connective tissue also had the characteristic oedematous appearance seen in the body of the tumours, and never extended beyond into the denser collagen of the bladder wall. Very occasionally in these areas one could find tumour cells apparently invading thin walled blood vessels (fig. 59).

vi. Deep invasion, down to or into the muscle of the bladder wall, was first seen at week 22. In this instance and on subsequent occasions when this was found the infiltrating cells were seen either singly or in small groups (figs. 60, 61, 62 & 63). Invasion of this type was found in only 3 of 14 animals sacrificed between week 22 and the end of the experiment. One of these was at week 22 and the other two were seen at week 35.

vii. Metastatic tumour was only found on one occasion - in an animal sacrificed at week 35. This secondary carcinoma was present in the left inguinal region. Microscopically it was an undifferentiated carcinoma, composed of cells resembling those found in the infiltrating portion of the carcinoma of the bladder (figs. 64 & 65), but there were areas

similar to a poorly differentiated squamous carcinoma.

Electron microscopy was of no help in identifying early invasion. At no stage were defects in the basal lamina found. Cell groups lying deep in the stroma of invasive carcinomas were completely surrounded by an uninterrupted basal lamina.

Squamous epithelium, in the bladders of test animals, was first noted at week 11. This squamous metaplasia was deemed to be present when the cells showed one or more of the following features: intercellular bridges, keratohyaline granules or intra or extra cellular keratin. At week 11 squamous metaplasia was found in small stage 2 lesions of the nodular type where it formed the central part of the lesion (fig. 66). In this central situation the full thickness of the hyperplastic epithelium was composed of squamous cells and was capped on the surface by keratin. Two lesions of this type were found in the experiment; both in animals sacrificed at week 11. A very similar lesion was found at week 19 which, while having the same configuration, was placed on the side of a papillary lesion (fig. 67). The cells of polypoid nodular tumours were commonly angulated and separated from each other by a widened intercellular space (fig. 68). This, with a mild basophilia in H&E stained sections suggested squamous metaplasia. No keratin and no keratohyaline granules were present. Although electron microscopy of these areas in general showed only widening of the intercellular space (as described above) there were parts which showed, in addition, an increased number of desmosomes and prominence of tonofilaments which in some instances could be seen running into the desmosomes (fig. 72). In the terminal stages of the experiment, in some tumours which contained cells showing

similar light microscopic appearances there were prominent tonofilaments and increased numbers of desmosomes in the basal cells while in the cells away from the basal layer these features were unobtrusive (fig. 73). In two animals sacrificed at week 20 and in three at week 30 there were frank keratinising squamous lesions (fig. 70). These were either small verrucous lesions on the bladder wall or were part of one of the larger tumours (fig. 69). In this type of squamous lesion the mitotic activity was confined to the basal layers and there was an orderly maturation through a prickle cell layer to the keratin as seen in normal skin (figs. 70 & 71).

Inflammatory cells were present in the bladder walls of test animals but not in controls. From week 5 onwards the occasional test animal showed the presence of lymphocytes in a perivascular situation in the bladder wall not necessarily related to any particular epithelial abnormality. From week 15 onwards most bladders from test animals showed the presence of lymphocytes but seldom in great numbers. However, not all test animals showed this reaction and at any one week some test animals showed no lymphocytic infiltration at all. This lymphocytic infiltration was in many instances found at the base or in the core of the tumours or indeed admixed with the epithelial cells but lymphocytes were also found in areas of the bladder wall far removed from localised epithelial abnormality (figs. 74 & 75). There was no relationship between the type of tumour and the lymphocytic infiltration. There was no relationship between invasion and the presence of lymphocytes. Very rarely labelled lymphocytes could be found in the autoradiographs.

Mast cells were present in small numbers in the urinary bladders of control rats. In the test animals, at week 18, moderate numbers of mast cells could be found at the base of tumours and after week 22 these mast cells were very numerous and were scattered throughout the tumours (figs. 76 & 77). The mast cells were recognised by Giemsa stain but were also found to contain abundant acid mucopolysaccharide which was stained by alcian blue. Other inflammatory cells seen in the test animals' bladders, but not in those of the controls, included polymorphonuclear neutrophils, particularly in areas of necrosis or ulceration and the occasional eosinophil.

4. The Mitotic Activity of Developing Tumours.

Examination of the autoradiographs suggested that the nodular lesions contained a greater percentage of labelled cells than the papillary lesions. Attempts were made to assess a labelling index for each of these two types of lesion as described above in the section on materials and methods.

In stage 2 lesions (calculated in animals sacrificed at week 7) difficulties were experienced due to the small size of many lesions - mainly the micropapillae - so that many of them contained no labelled cells. In addition a few of the nodular lesions contained no labelled cells. Therefore, the standard deviations of the mean labelling indices for the two groups were relatively large. The mean labelling index for the 14 nodular lesions assessed was 1.3% and the mean labelling index for the papillary lesions examined was 0.3%. When tested by chi square this difference proved to be statistically significant ($p<0.01$). Most of the labelled cells in both these lesions were seen in the basal layer.

Due to the morphology of the two lesions the ratio of the basal layer cells to the total number of cells was greater in the papillary lesions. Assessment of the labelling indices of the basal layers of each of these two lesions showed a mean index for the nodular lesions (basal cells) to be 2.3% and that of papillary lesions 0.4%. This difference was statistically significant by chi square ($p < 0.01$.).

In assessing early stage 3 lesions (calculated on animals sacrificed at week 15) combined lesions were excluded. Only 4 pure nodular tumours and 3 pure papillary tumours were found suitable for examination. The mean labelling index of the nodular tumours was 13.6% and that of the papillary tumours 4.1%. Tested by chi square this difference was statistically significant ($p < 0.01$.). When the basal layers only were examined the mean labelling index for the nodular tumours (basal layer) was 18% and that of the papillary tumours 4.5%.

DISCUSSION

The use of an experimental model to study the morphology of developing neoplasms with a view to relating the findings to the human situation may be criticised in that no animal tumour can be said to be identical to the human counterpart. While there are many similarities between the lesions shown herein and human bladder tumours no direct comparison is implied in this discussion. However, when dealing with the broader concept of histogenesis rather than morphologic detail some comparison may be valid. Attempts will be made to show that the changes described in this experiment do in fact have a wider relevance than that to the model in which they have been demonstrated. Further discussion will centre around the different aspects of tumourigenesis and related phenomena.

1. The Validity of the Model

The morphologic changes related to tumourigenesis in the urinary bladder described in this experiment are not unique to this model. Ito and his co-workers [71] observed similar changes in the rat urinary bladder on administering N-butyl-N-(4-hydroxybutyl)nitrosamine. Hueper et al[68] and later Scott and Boyd[118] have illustrated nodular lesions in the dog's urinary bladder on feeding 2-naphthylamine and Heuper et al describe papillary outgrowths which can be regarded as the same as the micropapillae recorded here. The tumours found in the monkey bladder on feeding 2-naphthylamine[31] are similar to the tumours described in this report. The changes produced in the mouse bladder by the implantation of pellets containing various carcinogenic agents are somewhat different but do show some similarities[16,115]. Bonser and Jull[16] refer

to telangiectasis of capillaries and lymph vessels in areas of hyperplasia. These authors and also Roe[115] illustrate downgrowths of epithelial cells to form van Brunn's nests. These downgrowths, however, appear to be occurring in an otherwise non-hyperplastic epithelium. Neither of these reports[16,115] indicate the presence of micropapillary outgrowths and although Bonser and Jull refer to transitional cell papillomas they state that these are "usually sessile but may be pedunculated". The differences noted between the implantation studies and those where the carcinogen is administered by mouth may be due to no more than the presence of a foreign body with the bladder lumen.

In the human bladder microscopic examination of the cystoscopically normal epithelium of bladders containing overt carcinomas has shown the presence of nodular downgrowths of epithelium[95,120]. Temkin[123], who had the opportunity of routinely examining workers exposed to industrial bladder carcinogens describes the development of neoplasms in the human bladder. Temkin's work and descriptions are mainly cystoscopic and are supported by relatively little histologic data but a brief review of his findings is indicated. He divides the lesions seen cystoscopically into six types viz. 1. "the vascular clump", 2. "foci of intense hyperaemia with cellularity and honeycombing"; 3. "foci of hyperplasia and roughness of the mucosa"; 4. "nodular sub-epithelial" lesions; 5. "early forms of papillary neoplasm" and 6. malignant and infiltrating neoplasms. The vascular clump, which is a focal area of richly developed capillaries, was not studied histologically but was regarded as the earliest pre-neoplastic change because, with repeated observations, some of these lesions regressed while others progressed to

neoplasia. Lesions in groups 2, 3 and 4 were examined histologically. Nodular hyperplasia (called by him "commencing papilloma", "submergent growth" and possibly also the "nodular sub-epithelial" lesion) and micropapillae ("early papilloma" and "micropapillomatosis") are described as the early neoplastic changes. These lesions are similar to the early changes described in this report. That the earliest pre-neoplastic change found cystoscopically in occupational bladder tumours is a vascular rather than a epithelial change is also noted by Simon[119] and Gay[59]. Gay also describes an initial proliferation of the basal epithelial layer so that there is a downgrowth of cells into the bladder wall. The "papillary hyperplasia" described by Eisenberg et al[47] is very similar to the early nodular hyperplasia found in the experiment described in this report.

The preference for a continuous feeding experiment, as used here, rather than a discontinuous one needs to be commented upon. The aetiology of most human bladder cancers, those unassociated with an industrial exposure, is largely unknown. Among others there are statistical associations with smoking[28,43,53,74,85,122,136] and coffee consumption[27,43] as well as with abnormalities in tryptophan metabolism[18,107,108]. In many instances these factors continue to operate after the initial development and treatment of the bladder neoplasm and may aggravate the condition[7]. For the purpose of studying histogenesis, therefore, continuous feeding experiments were regarded as more legitimate than a discontinuous regime.

From the foregoing it is concluded that the model used is a legitimate one for the study of the histogenesis of bladder tumours in general and further that the discussions of the histogenesis of the experimentally produced bladder

tumours may have some relevance to the human disease.

2. The Vascular Response.

The first morphologic change noted in this experiment was a vascular response. That this increased vascularity may be partly due to the dilatation of pre-existing capillaries and arterioles cannot be disputed but there is autoradiographic evidence to show that there is also endothelial proliferation. Similar endothelial proliferation has also been noted by Dzhioev et al[45] after giving 4-ethylsulphonylnaphthalene-1-sulphonamide to mice for 4 weeks. This vascular response may be the result of one or more of three factors: a. the direct action of the carcinogen or its metabolites on the blood vessels; b. a direct response to changes occurring in the bladder epithelium or c. the action of an unknown substance released by a distant organ as a result of a. or b. above.

a. The direct action of the carcinogen or its metabolites may be toxic or pharmacologic. Increased vascularity as a result of direct toxicity is seen, for example, in the early stages of cyclophosphamide damage to the rat bladder epithelium[22,75,125]. The action of drugs on the bladder vasculature has been studied by Matsumura and his co-workers[90] who have shown that a wide variety of drugs that cause constriction of vessels elsewhere in the body may cause dilatation of the vesical vessels. It is not known whether or not N-(4-(5-nitro-2-furyl)-2-thiazolyl)formamide (FANTF) or one of its metabolites has a similar toxic or pharmacological effect. The direct action of these chemicals on the vesical vasculature may be via the systemic circulation or may be via the excreted urine by absorption through the bladder epithelium.

While barriers to the permeability of transitional epithelium to water have been demonstrated[63] other studies on the transport mechanisms of bladder epithelium have established that a wide variety of substances may be absorbed through the bladder wall[21,88]. Further, Conklin and Hollofield[29] have shown that nitrofurantoin, a chemotherapeutic agent with a chemical structure similar to FANTF, is readily absorbed through the bladder epithelium. It is evident, therefore, that either of these two routes may apply.

b. The second possible cause of the vascular response is that it follows a change in the epithelial cells of the bladder wall. Light and electron microscopy have shown only little morphologic change in these cells at this early stage but a greater change at the molecular level cannot be excluded. Algire and Chalkley[3] have suggested that one characteristic of malignant cells is their capacity to provoke a continued vascular proliferation. This stimulation by malignant cells of endothelial proliferation has been shown to occur in the hampster cheek pouch when a malignant tumour, contained in a Millipore filter chamber, is inserted [46,60]. Folkman et al[52] have isolated a substance called by them tumour angiogenesis factor, which is liberated by tumour cells and which is mitogenic to capillary endothelial cells. In all these instances, however, the cells studied were obtained from obvious malignant tumours. Because it has been shown[125] that the early changes (week 4) in the present experimental system of bladder carcinogenesis are reversible, it is doubted that the epithelial cells are truly malignant at this stage. Similarly Temkin[123] has stated that the "vascular clump" lesion may return to normality. Nonetheless one cannot discount the possibility that the epithelial cells of the bladder are producing the angiogenic

factor at this early stage of carcinogenesis.

c. The third possibility is that the increased vascularity is due to the action of some unidentified substance released by a distant organ under the influence of the carcinogen. Ertürk[48] has shown that when labelled FANTF is fed to rats the label can be recovered in high concentrations from the intestinal contents, the urine, the bladder wall and the adrenal glands. No morphologic change has been noted in the adrenals in these experiments but the possibility of interference with one of several endocrine systems cannot be discarded.

3. Early Hyperplasia and Mitotic Activity.

Hyperplasia is the result of an increased cell proliferation unbalanced by an equal increase in cell loss. In this model increased proliferation is shown by the increased number of labelled cells seen in the autoradiographs. Increased proliferation has also been demonstrated by using the stathmokinetic agent colchicine[127]. Data not included in that report[127] have shown that, within the limitations of the colchicine technique[12] rough doubling times for the overall bladder mucosa at week 8 should be 500 hours and for tumours seen at week 12, 200 hours. Subjective observation suggests that the tumours did not increase at that rate. Explanations for this discrepancy may be found in experimental error but another reason for the less than expected rate of growth is the presence of increased cell loss. That there is an increased number of exfoliated cells in the urine of test animals has been confirmed by the microscopic examination of voided urine of both test and control animals[125]. These exfoliated cells presumably arise from the surface of the

epithelium. The cells lost from the surface must then be replaced by the underlying cells and it has been shown how, in the hyperplastic epithelium, the underlying cells push through to the surface between the superficial cells.

This report contains only a few facts concerning the kinetics of the mitotic activity of the described lesions but there are a few points of interest. A mitotic index calculated in a single pulse labelling with tritiated thymidine does not strictly indicate proliferative activity because the technique only identifies cells in the synthetic phase of the mitotic cycle. Because it has been shown both here and elsewhere[127] that there is indeed a true increase in the number of mitotic figures in these lesions the increased labelling index found in this experiment may be regarded as an indication of the degree of mitotic activity. In this experiment the epithelial mitotic activity is virtually confined to the basal layers until fairly large tumours are present where it can be found throughout. This is of interest in that in the normal rat urinary bladder mitoses are found in all cell layers[80,127]. Walker[131] has shown that the regeneration of the mouse bladder epithelium after mechanical trauma involves all cell layers. Levi et al[83] have shown that when the mouse urinary bladder is stimulated with 4-ethylsulphonylnaphthalene-1-sulphonamide the initial mitotic activity involves all cell layers equally but in the later stages of their experiment the mitotic activity was found mainly in the basal layers but all cell layers always showed some mitotic activity. That there may be some species differences in this regard is suggested by the work of Martin[89] who demonstrated that when the guinea-pig's bladder was acutely distended the resultant wave of mitotic activity was confined to the basal layers. The bladder

epithelium of many different mammalian species is polyploid[32,82,83,86,130]. Cells of higher ploidy are found in the superficial layer while the smaller cells are in the basal or intermediate layer[45]. Levi et al[82] have shown that bladder tumours appear to originate as diploid tumours. Does this not suggest that they arise from the basal layers, that area where the bulk of the mitotic activity is found?

4. Focal Hyperplasia.

The localised areas of increased hyperplasia, presumably the result of focal proliferation, have been shown to have an exophytic or an endophytic pattern. Both types were seen in the same bladder and were seen together in many instances. It is further assumed that these localised areas of hyperplasia were the forerunners of the larger tumours. The endophytic growth would appear to be the more logical type of hyperplasia. As has been shown the bulk of the proliferative activity is seen in the basal layers of the epithelium in contrast to the normal or regenerating bladder epithelium where it is seen in all cell layers[113,131]. It has also been shown in the mouse urinary bladder that the initial proliferative response after ingestion of 4-sulphonylnaphthalene-1-sulphonamide involves all cell layers but the prolonged response is predominantly in the deeper levels[24,83]. Mitotic activity in this region can easily force cells into the bladder wall. Further, the hydrostatic pressure within the bladder lumen, in the absence of elastic tissue or a muscularis mucosa, is maintained by the muscle deep in the bladder wall[5]. This being so, the interface between the two forces - the hydrostatic pressure and the muscular action maintaining the pressure - lies deep to the epithelium. Would not this direction of force favour growth

into the bladder wall rather than into the lumen?

The production of the papillary outgrowths is more difficult to understand. Division of cells parallel to the basal lamina may result in an outfolding of the full thickness of the epithelium. Such parallel divisions were observed in the study of telophases in papillary lesions but not in the nodular type. Nonetheless, in view of the discussion above outfoldings into the bladder lumen would appear a less likely occurrence than a bulging down of the base of the epithelium into the underlying bladder wall. Three possible explanations for this outfolding exist. Endothelial cell proliferation is present in the bases of the micropapillae and it is possible that these papillae may be partly produced by the pushing effect of the growing capillaries destined to form part of the fibro-vascular core of the lesion. Secondly, the outfoldings may be related to the relatively slow rate of growth, as indicated by autoradiography. Because of the slow increase in the number of cells the periodic contraction of the bladder may have a greater moulding effect, by buckling the lesion into the bladder lumen, than where this increase is more rapid. Thirdly, the papillary outgrowths may be due to a more localised area of cell proliferation. The presence of a nodular mass of cells deep to the epithelium over a wider area would, due to its anchoring effect, preclude formation of a papillary lesion at precisely the same site. The presence of outfoldings in the combined lesions is due to the manner in which the epithelial cells grow down into the bladder wall, often beneath the surrounding epithelium and lift up the adjacent epithelium thus initiating a direction of growth into the bladder lumen. In this instance the papillary outgrowth

occurs adjacent to the nodular downgrowth. This explains why many of the combined lesions had a papillary structure at the periphery and a nodular structure centrally. It is also possible for more complex lesions to arise by two or more nodular downgrowths squeezing up the intervening epithelium towards the lumen. Once the lesion has been initially formed further growth, following the path of least resistance, would continue in the same direction.

5. Malignancy.

In this report the term carcinoma has been reserved for those tumours showing true invasion. It is, therefore, important to be able to define true invasion. Willis[134] defines such invasion as an insinuation of tumour cells into the interstices of the stromal tissues and differentiates it from expansile growth. In this study two forms of encroachment have been described - expansile and infiltrative. It is the identification of the latter that has been required for the diagnosis of malignancy. Expansile growth was well seen in the nodular hyperplastic lesions and in the polypoid tumours. It was also, but less well, seen in the bases of papillary lesions after week 15. Friedman and Ash[56] noted similar expansile growth in human bladder tumours and considered it not as invasion but possibly an indicator of aggressive potential of the tumour. Infiltrative growth was first seen in nodular tumours, as spurs of tumour cells, after week 14. Similar spurs of cells were not seen in the papillary tumours of this experiment. Pugh[110] illustrates similar breakthroughs of epithelial cells at the base of a papillary tumour in the human which is regarded as early invasion.

Three other types of local invasion have been described in this study. That where the tumour cells are invading the bladder musculature can be regarded as undoubted invasion. The remaining two, although described separately are probably different manifestations of the same process - narrow bands of cells extending into the underlying tissue which, when cut transversely, may appear as isolated islands. The deep penetration of these protrusions certainly suggests invasion but they were never seen extending beyond the inferior border of the tumours. Because of this failure to extend beyond the base of the tumour one is left with some uncertainty whether or not this represents true invasion. The basal lamina, as seen in electron microscopy, was not broken. Tannenbaum et al[121] describing the electron microscopic evaluation of human bladder cancers has shown invading islands of epithelial cells in the connective tissues incompletely surrounded by a basal lamina. This finding was not demonstrated in this study but muscle penetration was not examined at the ultra-structural level. If one accepts that the basal lamina must be breached before invasion, of the type that is capable of entry into the tissue spaces, lymphatics and blood vessels, can take place then these narrow bands of epithelium do not qualify. On the other hand, tumour cells within the wall of a blood vessel, as illustrated in figure 59 must indicate invasion. The failure to demonstrate breaks in the basal lamina in the tissues examined does not preclude that such breaks were present elsewhere. Alternatively, even infiltrating epithelial cells may be capable of continued secretion of a basal lamina.

Invasion is not the only criterion that may be used in the identification of malignancy. It has only been used here to

positively identify carcinomas. The concept of carcinoma-in-situ is generally accepted as occurring in many organs and one can go further to state that all invasive carcinomas must have reached a point in time when they were committed to malignancy but had not realised their malignant potential by invasion. The term carcinoma-in-situ of the urinary bladder has been used to describe a generally widespread "flat" lesion of the bladder epithelium[93,97]. The term, might, however, also be incorporated into the papillary lesions of the bladder as papillary carcinoma-in-situ. It therefore seems obvious that in the experimental bladder cancer model some of the non-invasive epithelial tumours are indeed malignant. It has proved most difficult to recognise the cytological changes which constitute this malignancy. Increased nuclear size, increased nuclear/cytoplasmic ratio, clumping of chromatin, prominence of nucleoli and lack of differentiation are all cytological features associated with malignancy. However, most of the nuclear changes are also seen in regenerating bladder epithelium[126] and also in the very early stage of carcinogenesis in this study. Other studies[125] have shown that these very early changes (week 4) are reversible when the animal is removed from the carcinogenic diet, with return to normality after an additional 21 weeks. It has also been shown in the same study that after 14 weeks feeding the carcinogen the lesions produced are not reversible and continue to grow to produce invasive carcinomas after an additional 11 weeks. It has not been possible to identify any cytological differences on light and electron microscopy between the lesions seen at week 4 and week 14.

Another method that has been used to identify malignancy is the successful isogenic transplantability of tumour fragments.

Using FANTF induced tumours in male rats Ertürk et al[49] showed that tumours found after 52 weeks were transplantable. It has also been shown[124a] that tumours produced in male rats by feeding FANTF for 27 weeks are transplantable but those produced after 25 weeks feeding were not so. In this latter experiment, while it was not possible to examine microscopically the transplanted fragment, the remaining portions of the tumours showed features compatible with those found after a similar time period in this study. If then transplantability is considered a useful indicator of malignancy these findings suggest that such malignancy might only be present after the 25th week stage. This is in obvious conflict with the presence of stromal invasion at an earlier stage. Transplantability, therefore, must either be considered a poor criterion for the identification of malignancy or that it is subject to quite considerable experimental variation.

This inability to identify the difference between benign and malignant tumours has relevance to the human situation where this difficulty is constantly experienced with well differentiated papillary lesions and which was one of the motivations for this study. As has been already discussed correlation of morphology and behaviour in the human is complicated by treatment and also possibly continued carcinogenic stimulus. A more detailed assessment is possible in the experimental animal. Using the findings presented here as a base line, such investigations are being carried out[137].

6. The Stromal Component.

In this report most of the emphasis has been placed on the epithelial changes but connective tissues have also played a part in tumour development. In papillary lesions there is

only a very fine fibro-vascular core but labelled cells have been found in this tissue on autoradiography. The possibility that the outpocketing of the epithelium may be assisted by the pushing effect of the capillaries has been noted elsewhere. Connective tissues form a more important part of the nodular tumours where the configuration of the polypoid structure depends upon the stromal core. This core is made up of loose, oedematous connective tissue within which there are numerous blood vessels many of which are dilated. Mitotic activity is present in both the endothelial and other stromal cells and this may partly account for the increase. If this is so then one must conclude that either the carcinogen has a direct effect on the stromal tissue or that the altered epithelial cells have a mitogenic action, not only on endothelial cells, but also on other stromal elements. The first possibility is difficult to accept because the increase in connective tissue was only found in association with localised areas of epithelial proliferation. While it is difficult to explain why only localised areas of epithelium go on to produce tumours there was no increase in connective tissue associated with the generalised epithelial hyperplasia. Neither was there localised increase in connective tissue unassociated with a focal increase in epithelial proliferation. It is possible, on the other hand, that the resultant increased vascularity found in these areas favoured further epithelial growth thus producing epithelial tumours. Observations suggest, however, that the epithelial changes occur earlier than the increase in stromal tissue. That the epithelial changes promote stromal proliferation cannot be entirely excluded but it is difficult to understand why this should occur only in nodular polypoid lesions.

The initial increase in stromal tissue, however, was also partly due to oedema. Oedema and increased vascularity could be the result of local toxicity or of alteration in the epithelial cells. Once more it is difficult to understand why either of these should be more common in the nodular lesions and only appear late in the papillary tumours. An explanation that fits the observations better is that the oedema and the increased vascularity are only partly the cause but mainly the result of the polypoid structure. The bulging down into the underlying bladder wall of the nodular hyperplastic lesions may stimulate a local peristalsis in the bladder musculature which would force the lesion towards the bladder lumen. As the lesion is so directed it would pull with it the local connective tissue. As the tumour became more polypoid partial vascular stasis would result causing dilatation of the thin walled vessels and oedema of the surrounding stroma. This could also explain why the larger papillary tumours also had a similar oedematous stroma at the base. That is, when the papillary tumours grow in size they tend to draw the connective tissue at the bases with them in towards the lumen causing partial stasis.

While this may explain the early connective tissue changes it does not account for the appearance of the stroma in the filigree tumours seen late in the experiment nor does it explain the absence of such changes in the large, predominantly, epithelial tumours. While it is possible, and indeed likely, that there is stasis within the larger filigree tumours, causing oedema, many labelled cells were found in this tissue on autoradiography. Ertürk et al[49] have reported the presence of carcino-sarcomas in this model but in the present experiment there is no evidence to suggest that these stromal changes represent sarcomatous change. However, the characteristic

appearance of this filigree pattern and the manner in which the bands of epithelial cells appear to get thinner with increased duration of feeding (and increased size of the tumours) suggests that the stroma is an active component and despite its oedematous appearance may actually mould the epithelium into the characteristic narrow bands.

7. Anatomical Situation of Tumours.

Although in the terminal stages tumours were present on the lateral walls most of the tumours in this study were situated on the anterior wall and in the bladder fundus. These are the most dependent sites of the rat urinary bladder. Similarly Heuper et al[68] noted that in dogs fed 2-naphthylamine the majority of tumours were seen in the dependent parts of the bladder and not in posterior positions or in the trigone. Bonser[14], however, reports that 11 dogs treated with oral 2-naphthylamine showed individual variations in number and site of the tumours and that no general rule could be formulated. In the study of human bladder cancer reported by Kretschmer and his co-workers[78] 76.6% were situated in the trigone, the lateral walls involving the ureteral orifices or in the bladder neck region. In the upright human these would be the most dependent areas of the bladder. It is easy to understand why carcinomas should develop in the trigonal region in the human because this is where the carcinogen containing urine first reaches the bladder.

The observation that carcinomas or pre-malignant lesions are seen in the most dependent parts of animal bladder suggests that gravity may play a role. The contracted rat bladder shows some folding of the epithelium but the anterior and posterior wall are in contact unless separated by urine. When

there is only little urine in the bladder it will collect at the fundus and cause a more prolonged carcinogenic stimulus at this site. It is difficult to understand, however, why the anterior wall should be more susceptible to tumour production when both this and the posterior wall must by necessity be in contact with the carcinogen containing urine at the same time. It is suggested that urine collects first in the epithelial fold of the bladder epithelium and that this is more likely to occur on the supporting surface due to the action of gravity. In this manner, as in the fundus, the anterior wall receives a more continual stimulus. This situation is analogous to that where carcinomas develop in diverticulae of the human bladder to which there have been many references[1,17,66,81,91,98]. The development of increased localised, as opposed to generalised, hyperplasia may also be explained by this means although it is somewhat unlikely that the epithelial folds produced by bladder contraction would always occur at precisely the same site so that it was always the same area of epithelium that received the more prolonged stimulus.

8. Comparison of Endophytic and Exophytic Lesions.

Hicks[65] reported the loss of the asymmetry of the surface membrane in acute reaction to 4-ethylsulphonylnaphthalene-1-sulphonamide in the rat bladder and Levi and her co-workers[84] confirmed this finding in the mouse but also reported that 2-acetylaminofluorene, another carcinogen for the mouse urinary bladder, did not have the same effect on the surface membrane. That this may be no more than a toxic effect is shown by Koss[76] who reported a similar loss of AUM in rats following cyclophosphamide damage to the bladder epithelium. Fulker et al[58]

reported that in well differentiated human bladder carcinomas the surface membrane appeared normal but that in poorly differentiated bladder carcinomas this specialised surface membrane was absent. At no stage in the experiments reported here could one be certain that the surface membrane had lost its asymmetry. However, in endophytic lesions the surface of the superficial cells did not show the angular ridges seen in the normal rat bladder. These ridges were present in the exophytic growths. A similar loss of angulation has been noted in both well and poorly differentiated human bladder carcinomas[57]. The presence of angular ridges is a property of the normal asymmetric unit membrane of these cells and these ridges, in the normal rat bladder, are not eradicated by stretching[62]. It would seem, therefore, that although the surface membrane of the endophytic growths appears morphologically normal it has undergone some change so that it is incapable of maintaining an angular configuration. This change is apparently absent in the exophytic growths.

The fusiform vesicles, lined by AUM, found mainly in the superficial cells, near the luminal border, are either invaginations of the surface cut transversely and/or represent new membrane being transported to the surface from deeper in the cell[62,76]. The relative reduction in number of these fusiform vesicles in nodular lesions could then have two explanations. First, the reduction may be due to stretching of the cell so that the invaginations are taken up to allow this stretch to occur. This has been shown to happen in the normal bladder[114]. Second, the reduction may be because less AUM is being produced by the cell. While the degree of hyperplasia is greater in the nodular than the papillary lesions it is difficult to understand why the surface cell of the former would

require more stretching, and consequently more flattening of the surface invaginations, than the same cells of the papillary lesions. It seems more likely that the reduced number of fusiform vesicles is due to reduced production of AUM.

The intercellular junction is normally interdigitate in the bladder epithelium. In some areas of exophytic lesions this interdigitation was absent and the cell boundaries were straight. Fulker et al[58] reported a similar finding in poorly differentiated human bladder carcinomas. A more noticeable difference between the nodular and papillary lesions was the widened intercellular space in the former. Battifora et al[10] report a similar widening in non-neoplastic human bladder epithelium. Widening of this intercellular space is also reported or illustrated under several different experimental conditions[63,84]. In some instances this widening of the space may be artefactual but that it occurs in nodular and not in papillary lesions suggests that either the morphology of the nodular lesions allows this to happen more readily or that the cell membranes of the cells making up the nodular lesions are altered so that closed connections with the neighbouring cells are not present or are easily broken down.

These suggestions, that there is some alteration of the specialised surface membrane, that there may be some alteration of the cell membrane of the deeper cells and that the superficial cells are not able to produce sufficient AUM to form many fusiform vesicles all indicate that the cells of the nodular lesions are less well differentiated than those of the papillary type.

It has been shown that the proliferative activity of nodular lesions is greater than that of the papillary. The

statistical analyses of these results employed relatively crude methods and the statistical significance may be suspect. Yet a faster rate of growth for nodular lesions can be correlated with the suggestion that these nodular lesions are less well differentiated. It is postulated that cells using their energies for proliferation are unable to fully develop their cytoplasmic organelles. That poorly differentiated human bladder cancers have a higher mitotic activity than well differentiated tumours has been shown by Fulker et al[58] using a metaphase arrest technique and Battifora et al[11] and Veenema et al[128,129] using an in vitro H^3-thymidine labelling technique. On the other hand, Levi et al[82] could show no simple correlation between the H^3-thymidine labelling index and histologic type.

A third difference between the exophytic and endophytic tumours is the earlier occurrence of stromal invasion in the latter. The difference between expansile growth and true invasion has been discussed above. Both expansile growth and true invasion, no matter how defined, were seen at an earlier stage in the endophytic lesions than in the exophytic type. However, early invasion was not commonly found and the endophytic tumours were more common than the exophytic. It is possible, but unlikely, that the apparent absence of invasion in early papillary tumours was due to the selection of histological material. Serial sections of all preparations were examined to reduce this chance. In discussing the clinico-pathological behaviour of human bladder tumours Wallace[132] has shown that "solid" tumours are more often invasive and generally have a poorer prognosis than papillary tumours. While the differences between solid and papillary tumours in this context[110] is not strictly the same as the

endo and exophytic lesions described in this experimental model a broad comparison can be made.

What remains difficult to understand is why these different lesions should develop under identical conditions. That it is not a host difference is shown by the fact that they occur in the same bladder. This phenomenon may be related to the selective collection of carcinogen containing urine in certain epithelial folds of the bladder wall, as discussed above, so giving some areas a more prolonged, and intense, stimulation than others. This explanation seems a little tenuous. It may well be that the initial direction of growth is a chance occurrence and once established dictates the subsequent behaviour. While this may have some influence on the presence or absence of early stromal invasion it is less easily related to growth rate or degree of differentiation.

9. Squamous Metaplasia.

The presence of squamous epithelium in some of the tumours is regarded as a metaplasia. Non-neoplastic transitional epithelium is capable of a wide variety of proliferative and metaplastic processes[72,102] including squamous change. Hicks[63], however, believes that the cells lining the lower urinary tract are potentially keratinising cells which do not normally cornify. The presence of keratinisation in this situation could then be regarded as the result of prosoplasia. It is not proposed to discuss which of these mechanisms apply to this model. The term metaplasia is used to denote a morphologic change.

Squamous metaplasia has been observed in the human and animal bladder associated with infection[30,40,67,104], chronic irritation from foreign bodies[64,115], hypovitaminosis A[64,135]

and hyperoestrogenism[6] or other endocrinal disturbances[2] either alone or in combination. The extreme form of squamous metaplasia in the human bladder, referred to as leukoplakia, may be pre-cancerous with the development of squamous carcinomata[40,67] or may be associated with malignancy elsewhere in the lower urinary tract[30,112,117]. Outside areas of endemic schistosomiasis pure squamous carcinomas of the lower urinary tract compose a small percentage of bladder neoplasms[98,109] but transitional cell tumours showing areas of squamous change are more common. The suggestion is that pure squamous carcinomas may arise from epithelium which has undergone squamous metaplasia before tumour development while the mixed tumours have undergone squamous change afterwards. The present model conforms to the latter situation.

If the squamoid change seen in many nodular lesions is set aside for a moment, keratinising squamous metaplasia was an uncommon occurrence in this experiment being found on only 8 occasions. Three of these were in small hyperplastic lesions. The remaining were either verrucous tumours showing no indications of malignancy or were part of a malignant tumour showing, in addition, features other than squamous epithelium. Although these three types are different they may be considered together. Of the various factors, noted above, that have been associated with squamous metaplasia only infection cannot be discounted in this experiment. Pyuria was not a feature in these animals but the urine was never submitted for bacteriological examination. However, squamous metaplasia is often found in experimental bladder tumours. Discounting those situations where a foreign body is placed in the bladder squamous tumours have been described in several different models where the carcinogen is given by mouth or parentally[38,49,77,105].

It would seem, therefore, that the irritation or local toxicity of the carcinogen itself may be responsible for this squamous change. In this regard it is noticeable that, in this model, squamous metaplasia became more common with increased duration of feeding. Another explanation for this finding, which is also related to the increased size of the tumours, is that the squamous change is related to reduced blood supply with either relative anoxia or an accumulation of metabolites being responsible for the change.

In the limited extent to which squamous metaplasia presented here it was not possible to identify any associated behavioural significance. Invasion was not more or less common in squamous lesions. In a previous study[127] it was shown that there is no difference in mitotic activity between transitional or squamous cell tumours. Ertürk et al[49], however, have shown that squamous carcinomas produced in the rat urinary bladder by feeding FANTF are more easily transplantable than transitional cell carcinomas.

The squamoid change referred to above is the appearance seen in the nodular lesions. At the light microscopic level there was no keratin or keratohyaline granules but intercellular bridges seemed to be present. As has been shown by electron microscopy, this appearance was due to widening of the intercellular space with the "bridges" being cytoplasmic projections probably representing remnants of the normal interdigitation. On the other hand, there was a slight increase in the number of desmosomes and a prominence of tonofilaments which may indicate that this is an early form of squamous metaplasia. As above this change was not associated with any observable behavioural characteristics.

10. The Inflammatory Reaction.

That lymphocytes are present in the bladder walls of test animals and not controls suggests that their appearance is related to the carcinogen. Tumours in the human are often associated with a lymphocytic infiltration where it is regarded as a possible immune reaction. A similar finding in the bladders of these test animals may be interpreted in the same way. There is, however, no evidence in this experiment to further support the hypothesis. That the lymphocytic infiltration is not confined to the immediate vicinity of the tumours but is also seen in non-tumourous areas may only indicate that these other areas of epithelium are not normal and may represent a pre-malignant condition.

The presence of mast cells in the late stages of tumour development is interesting. There is considerable disagreement concerning the function of mast cells in both normal and pathologic tissue[116]. It is not proposed to discuss the possible roles of heparin, histamine and serotonin on the growth of tumours because this study presents no additional data. Nevertheless, the observations in this experiment support those of Bierich[13] and Cramer and Simpson[33] who have shown mast cells in the dermis during the experimental production of skin carcinomas in experimental animals and also of Dunn and Montgomery[44] who found an increase in mast cells in the underlying connective tissue of the uterine cervix in dysplasia, carcinoma-in-situ and to a lesser extent in invasive carcinomas of this organ. However, this finding contradicts that of Lascano[79] who, after studying many different epithelial and connective tissue neoplasms found increased mast cells only in basal cell carcinomas and pigmented naevi and also that of Gupta[61] who found a reduction of mast cells in human bladder and prostatic carcinomas.

CONCLUSIONS

The purpose of this work was to study the histogenesis of experimental bladder cancer, not only as an end in itself, but also to establish a base line for further studies. This is considered accomplished. Another consideration was whether or not such a study would assist in the understanding of the human disease. As has been shown, the histogenetic mechanisms described here may have some bearing on the development of tumours in the human bladder but no direct morphologic comparison is justified.

The initial epithelial changes noted during the development of carcinomas had a benign appearance. That these same benign looking lesions developed, with continued carcinogenic stimulus, into frankly malignant neoplasms is assumed but not conclusively proven because repeated follow up examinations of individual lesions was not performed. It is thought, however, that the assumption made is a valid one. In considering this thought, therefore, is it not relevant to consider that those lesions in the human bladder called papillomas may represent a pre-malignant change per se as well as being indicative of an "unstable" epithelium of the lower urinary tract? While the term "papilloma" as applied to an innocent looking tumour is histologically accurate, the supposition that it is a pre-malignant change suggests that there may be a better alternative title. The term "papillary hyperplasia" has been used elsewhere. While one appreciates the criticisms to the use of such a term that has also been used by different authors to describe different lesions, the same concept has been used in describing certain pathologies of the endometrium where hyperplasia merges with malignant

neoplasia. It is further suggested that an added description of the cytological atypicality, if present, would place any individual lesion at some specific point on the continuous curve between normality and frank malignancy from which some assessment of prognosis and decision on treatment could be made. A second possibility, to divide all papillary lesions of the bladder into groups, depending on cytological appearances, invasiveness etc., and to label them by a non-specific term such as "type", "lesion" etc. qualified by numerals or letters, may also be considered. This non-commitment has some merit but one would be required to define one's terms on each occasion they were used which makes this system less desirable than a descriptive one.

As many questions have been raised by this study as have been answered. I do not consider this to be undesirable; quite the reverse; for such is the nature of research. The studies reported here helped my understanding of human bladder cancer and I hope that the reader is similarly assisted.

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ILLUSTRATIONS

Figure 1. Normal rat bladder showing three layers of epithelial cells. The surface cells are stretched over the underlying cells (H&E X 450).

Figure 2. Normal rat bladder showing the manner in which the capillaries bulge into the epithelium (arrow, Reticulin X 450).

Figure 3. Normal rat bladder: Autoradiograph to show labelled cells in the basal layer of the epithelium (arrows) (H&E X 450).

Figure 4. Normal rat bladder: Autoradiograph showing labelled cell in the sub-epithelial connective tissue (arrow) (H&E X 450).

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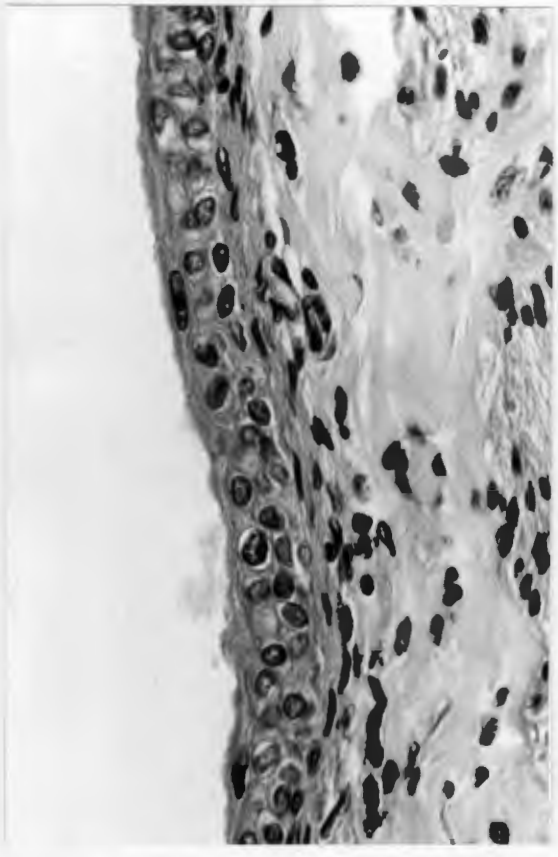

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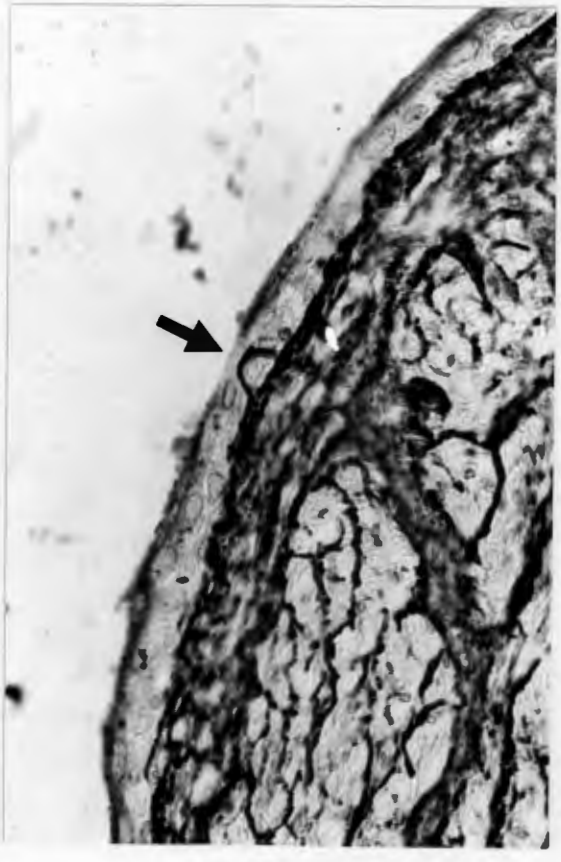

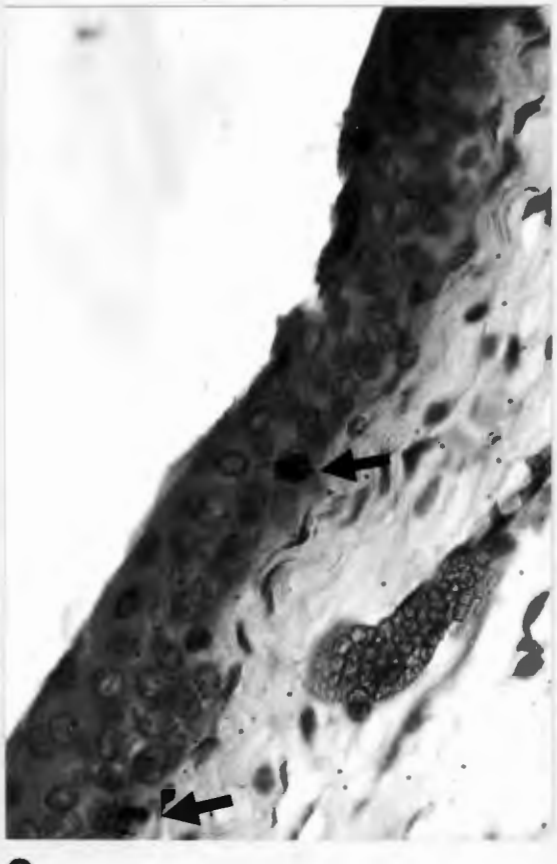

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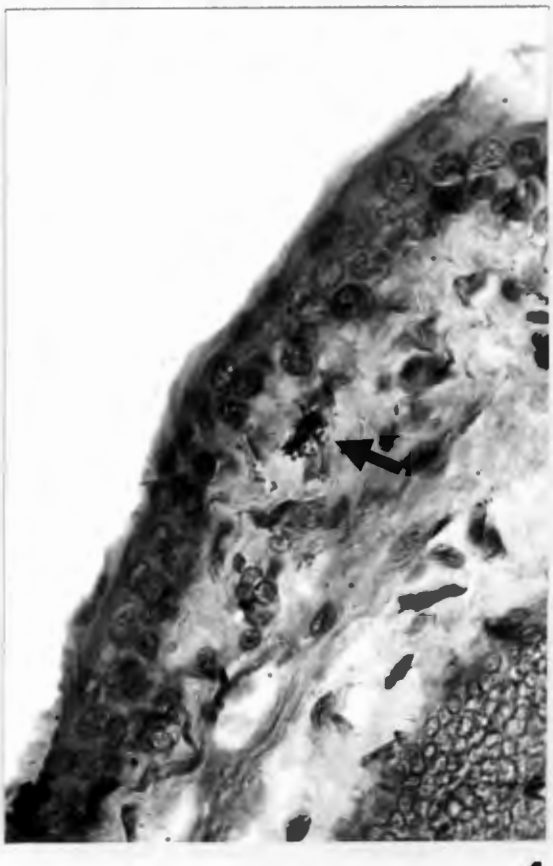

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Figure 5. Normal rat bladder (X 6000). The full thickness of the epithelium is shown. Below the basal lamina (arrow) there are two capillaries (c). The cells of the basal and intermediate cells show electron dense cytoplasm with small often indented nuclei. Numerous mitochondria (m) are shown in these cells. The intercellular junctions are markedly interdigitate and best shown on the left. The surface cells are usually relatively electron translucent but a darker surface cell is seen at the top left. The nucleus of the superficial cell is large and round. Telolysosomes (tl) are present. At the lumenal aspect of the cell are fusiform vesicles (f). The surface is thrown into angular ridges. The tripartite junction (t) between the adjacent cells is not clearly seen. Inset (X 150,000) shows the asymmetric unit membrane with the thicker component on the lumenal side.

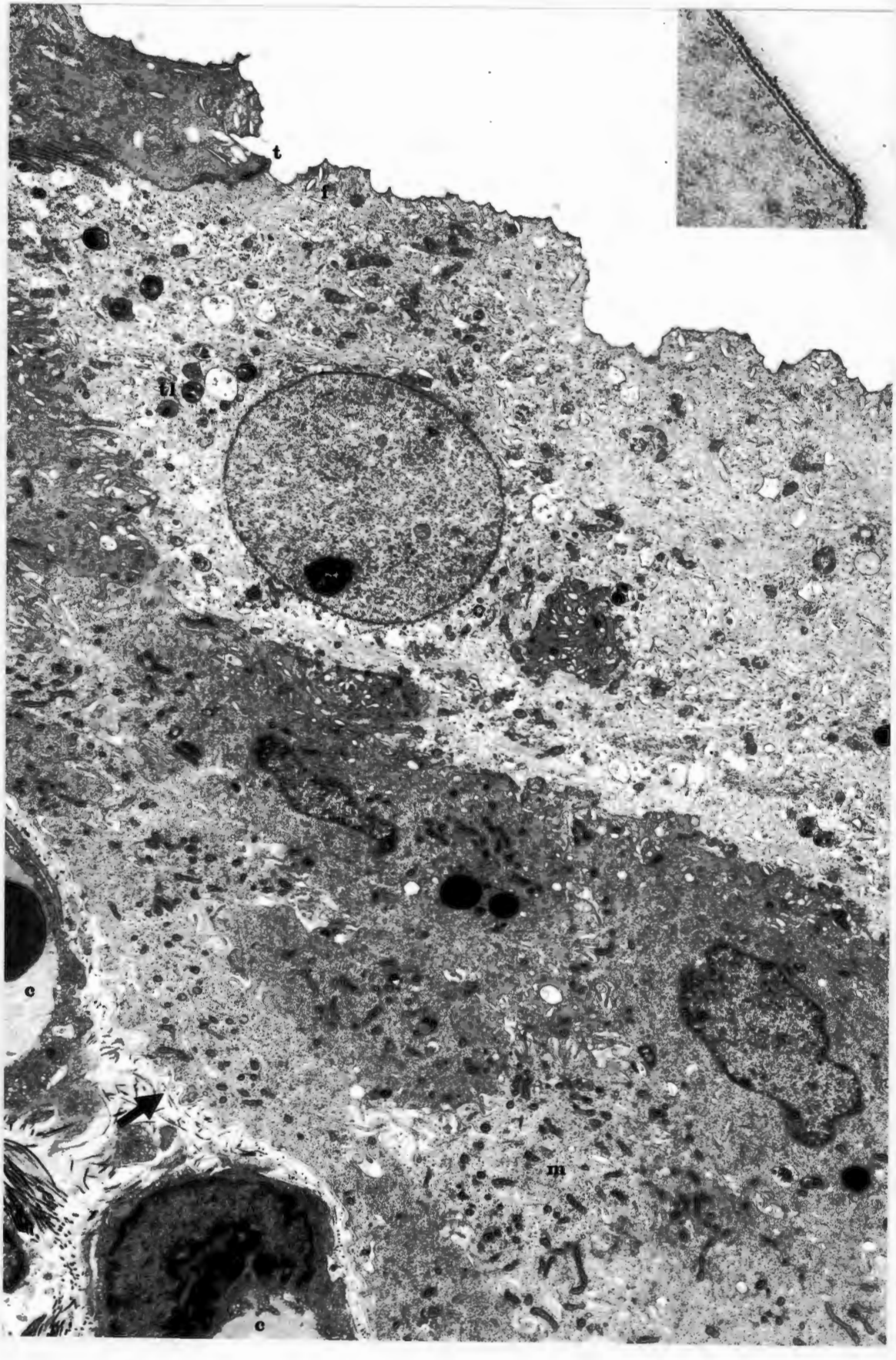

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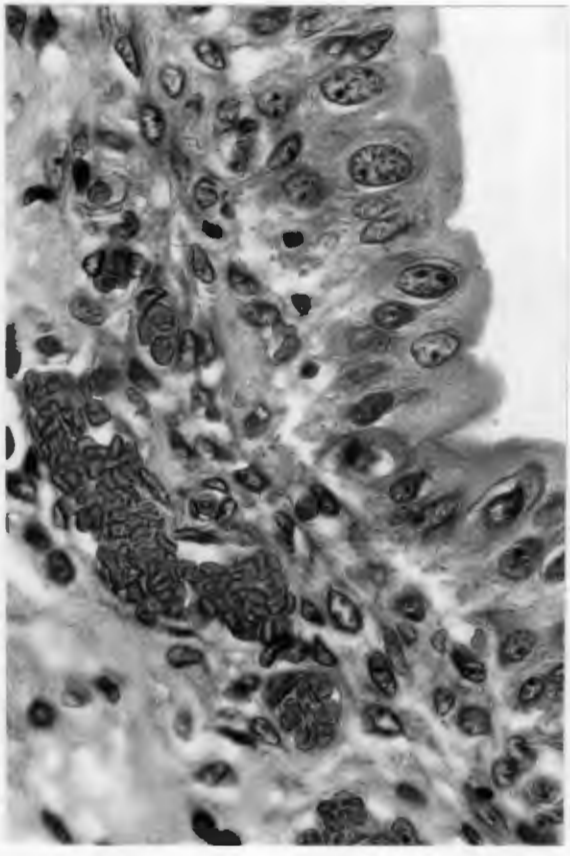
Figure 6. Prominence and congestion of small blood vessels in the sub-epithelial connective tissue. The epithelium shows nuclear changes characterised by enlargement and chromatin clumping (H&E X 600)

Figure 7. Prominence and congestion of arterioles in the sub-epithelial connective tissue and of capillaries at the epithelial/stromal junction. (H&E X 600)

Figure 8. Autoradiograph (week 3) showing labelled cells in the basal epithelial situation and labelled endothelial cells within the bladder wall (arrow) (H&E X 450).

Figure 9. Slight hyperplasia of the epithelium with increased number of cell layers. The nuclei show increased size, chromatin clumping and some pleomorphism. (week 4) (H&E X 600).

6



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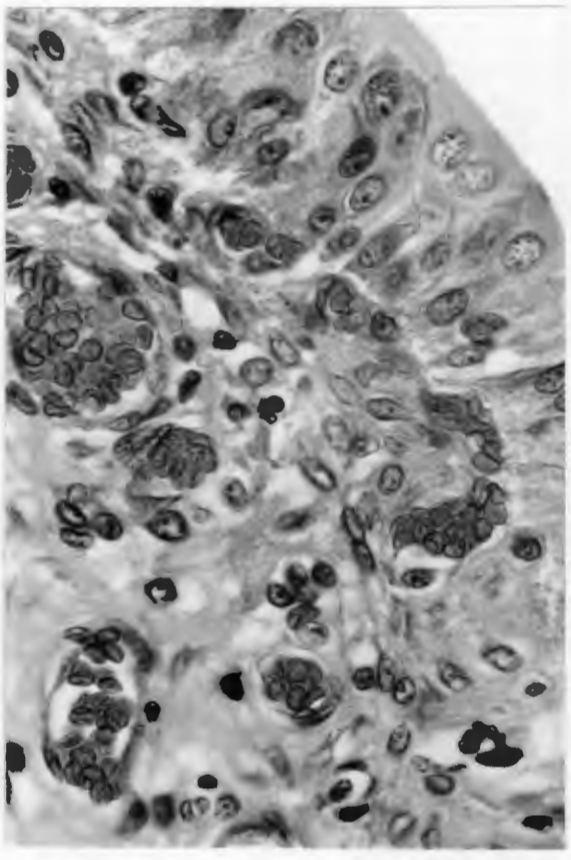

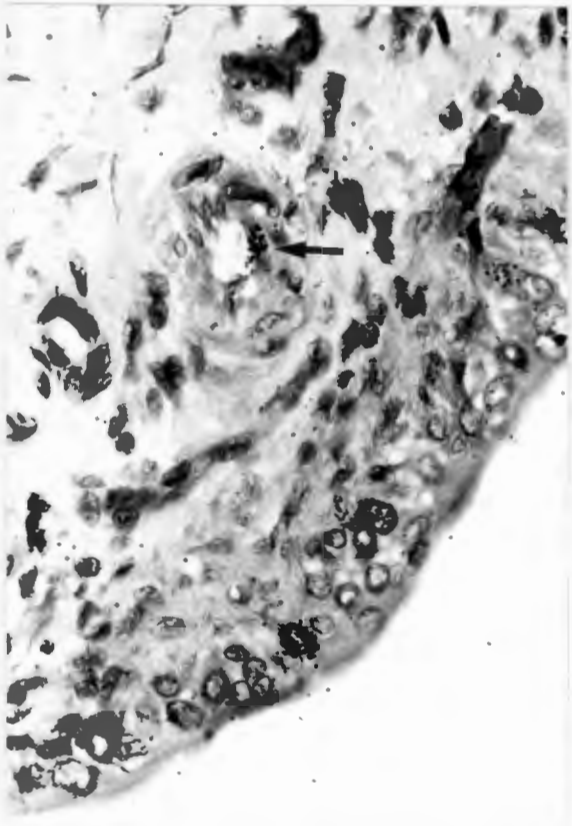

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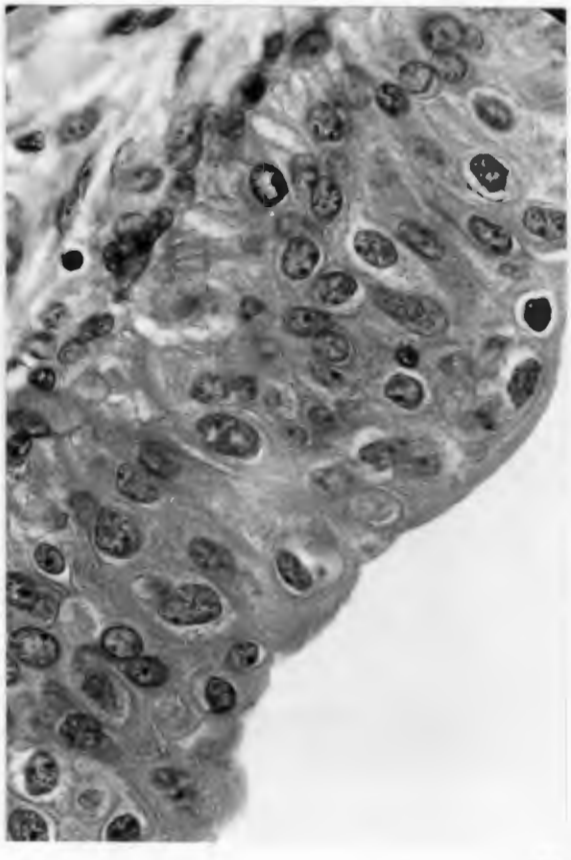


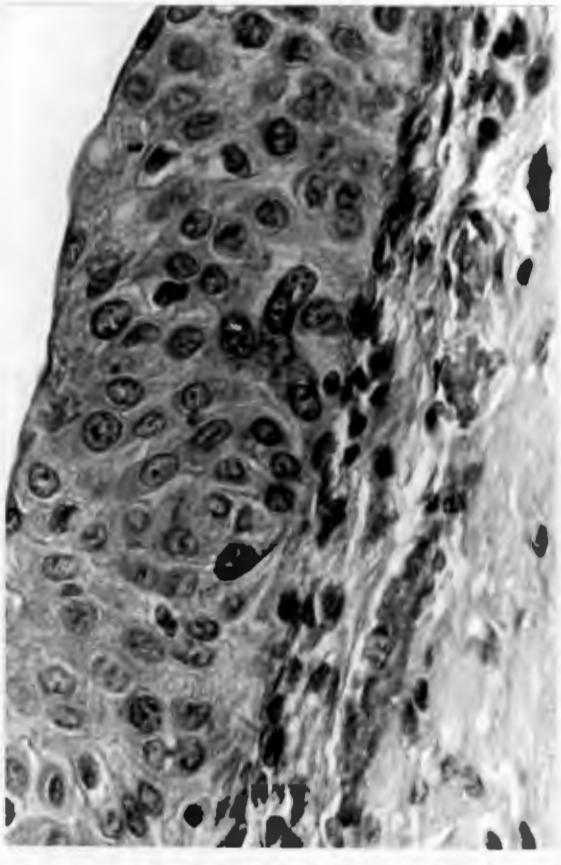
9

Figures 10 & 11. Localised areas where the normal base to surface differentiation has been replaced by groups of cells appearing very similar to each other. Note that in these areas (fig. 10) the nuclei appear somewhat different to those in the adjacent epithelium and that the cytoplasm stains darker. (H&E X 450).

Figure 12. At the basal area there is a small group of cells showing similar nuclear and cytoplasmic appearances to these cells in the small groups seen in figs. 10 and 11. The more superficial cells do not show this appearance. (H&E X 600).

Figure 13. Autoradiograph (week 7) showing the labelled cells in a basal situation. Note also a single labelled connective tissue cell in the immediate sub-epithelial position. (H&E X 450).

10



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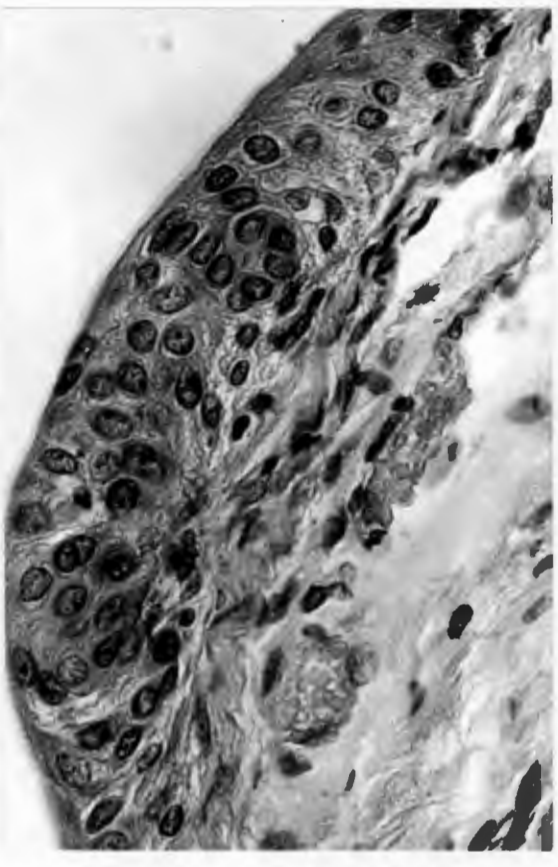

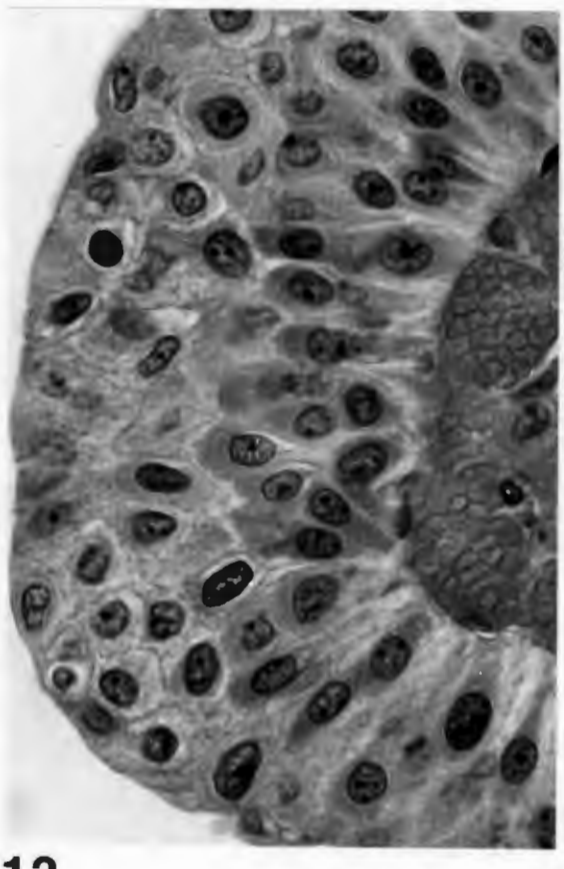

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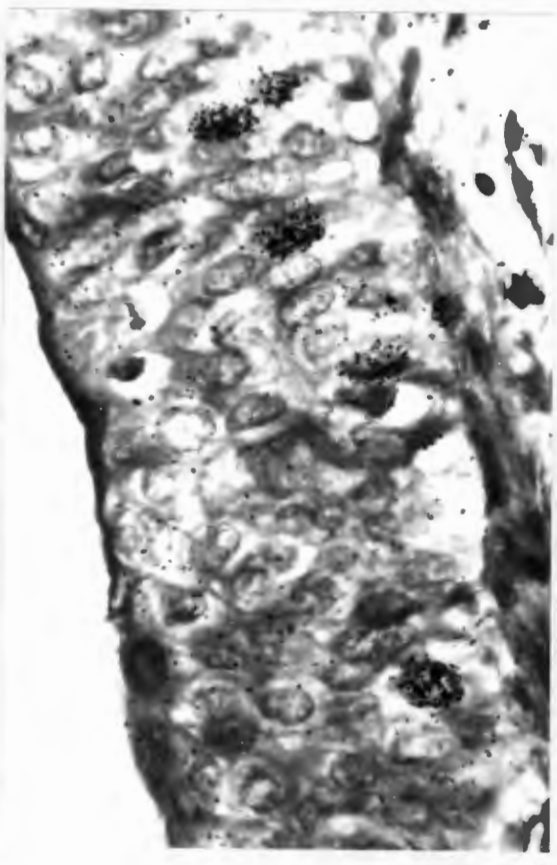

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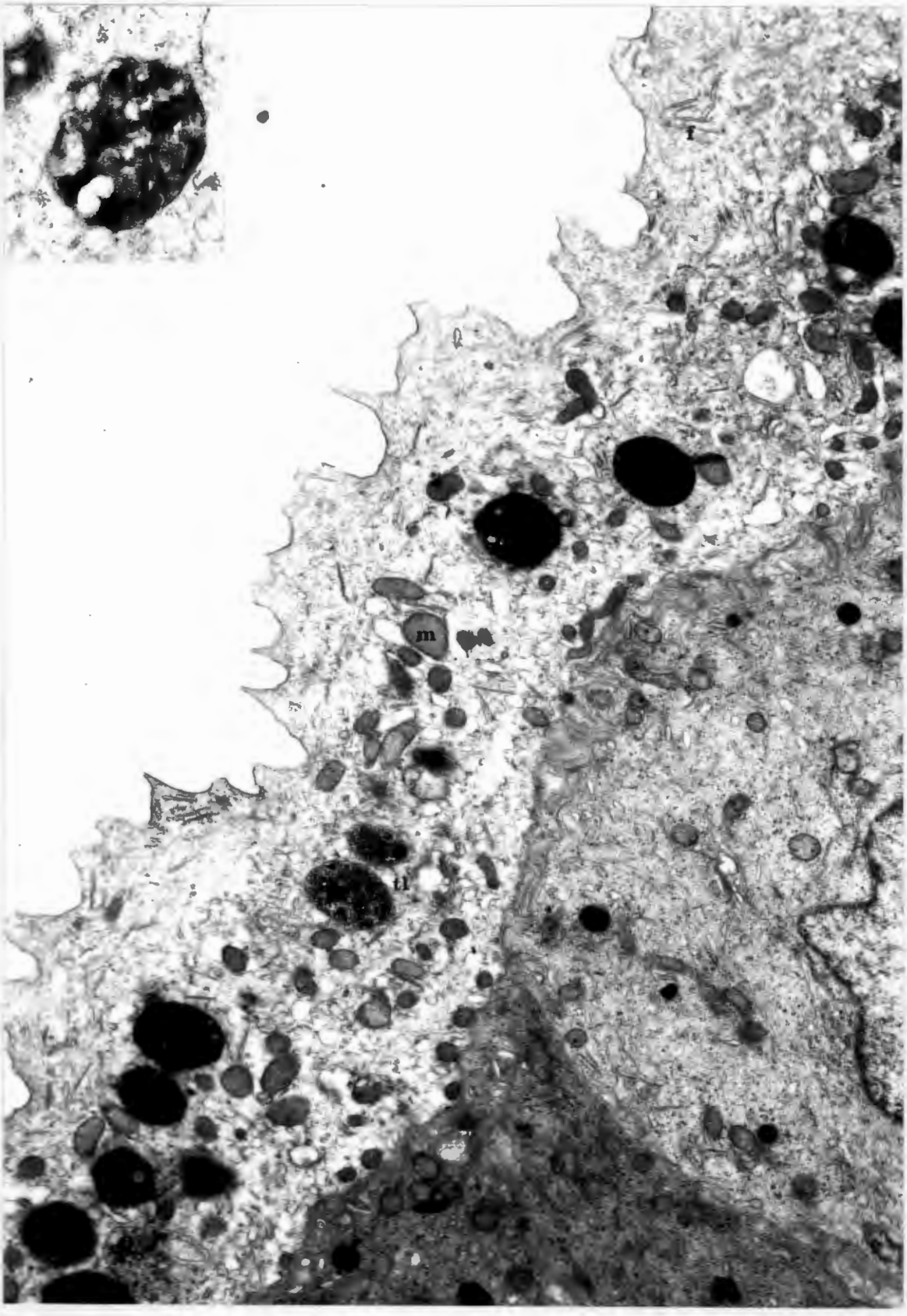
Figure 14. (X 20,000). The superficial cell appears stretched and flatted. Numerous teloly-sosomes (tl) are present but reproduce poorly because of the marked contrast in density between them and the cytoplasm. (The inset (X 40,000) shows the internal structure of these dark bodies. Electron dense and electron translucent sub-units are present, many of which have a hexagonal shape). The surface of the cell retains its angular ridges. Fusiform vesicles (f) appear mainly as slits. The mitochondria (m) present show few cristae.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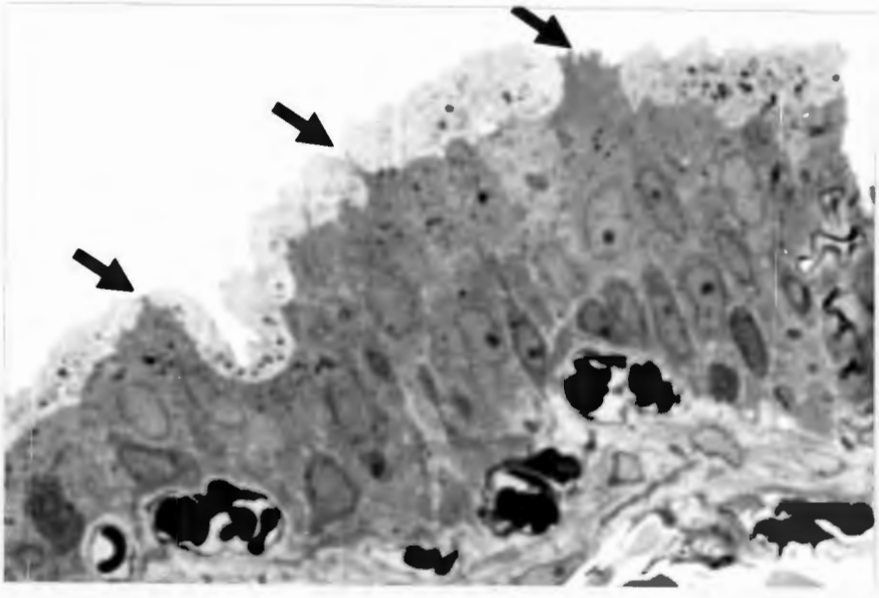
tl

Figure 15. Toludine blue stained "thick" section cut from araldite embedded material. Within this minimally hyperplastic epithelium dark cells can be seen intervening between the light superficial cells (arrows). The stained bodies in the light superficial cells are telolysosomes. (X 200).

Figure 16. (X 6000). This shows the same process as seen in fig. 15. The density of the cytoplasm and the shape of the nucleus suggests that these intervening cells come from the intermediate cell layer. The surface of these darker cells shows, in areas bizarre, angular ridges.



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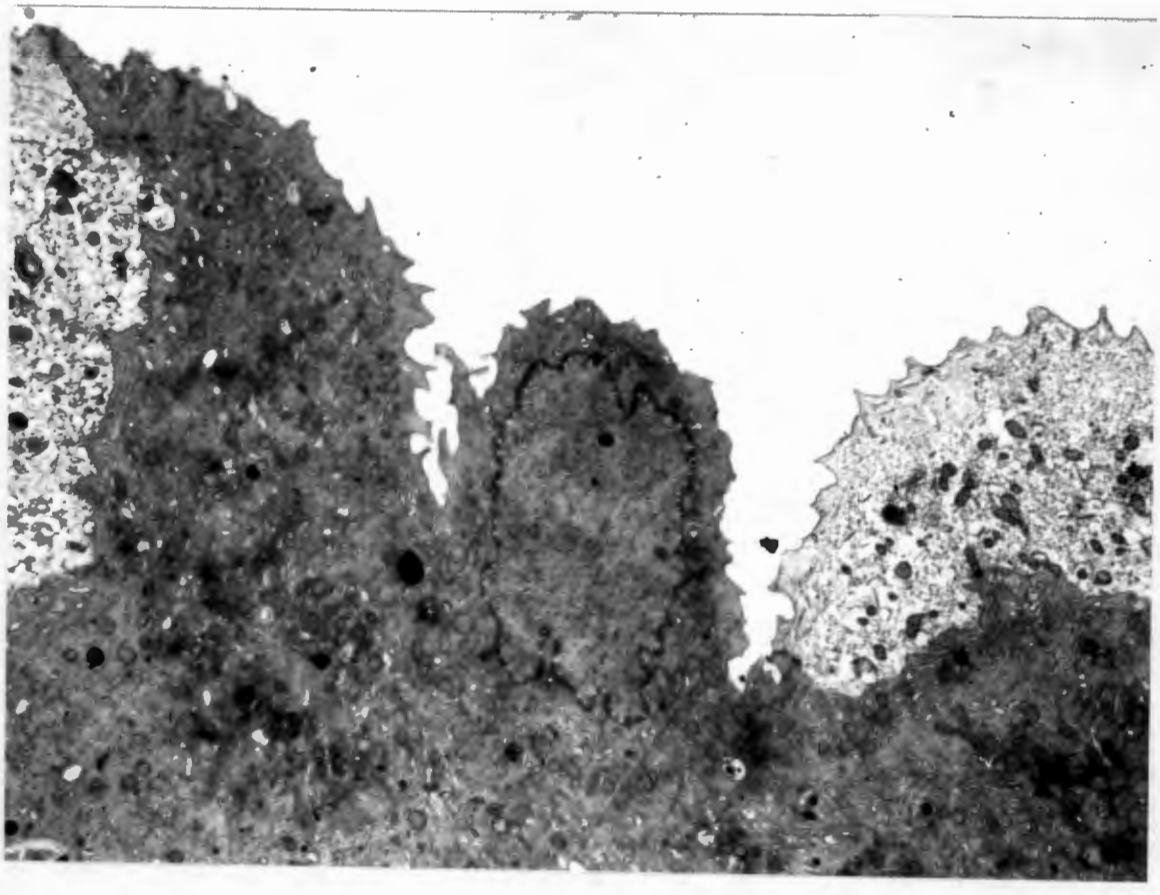
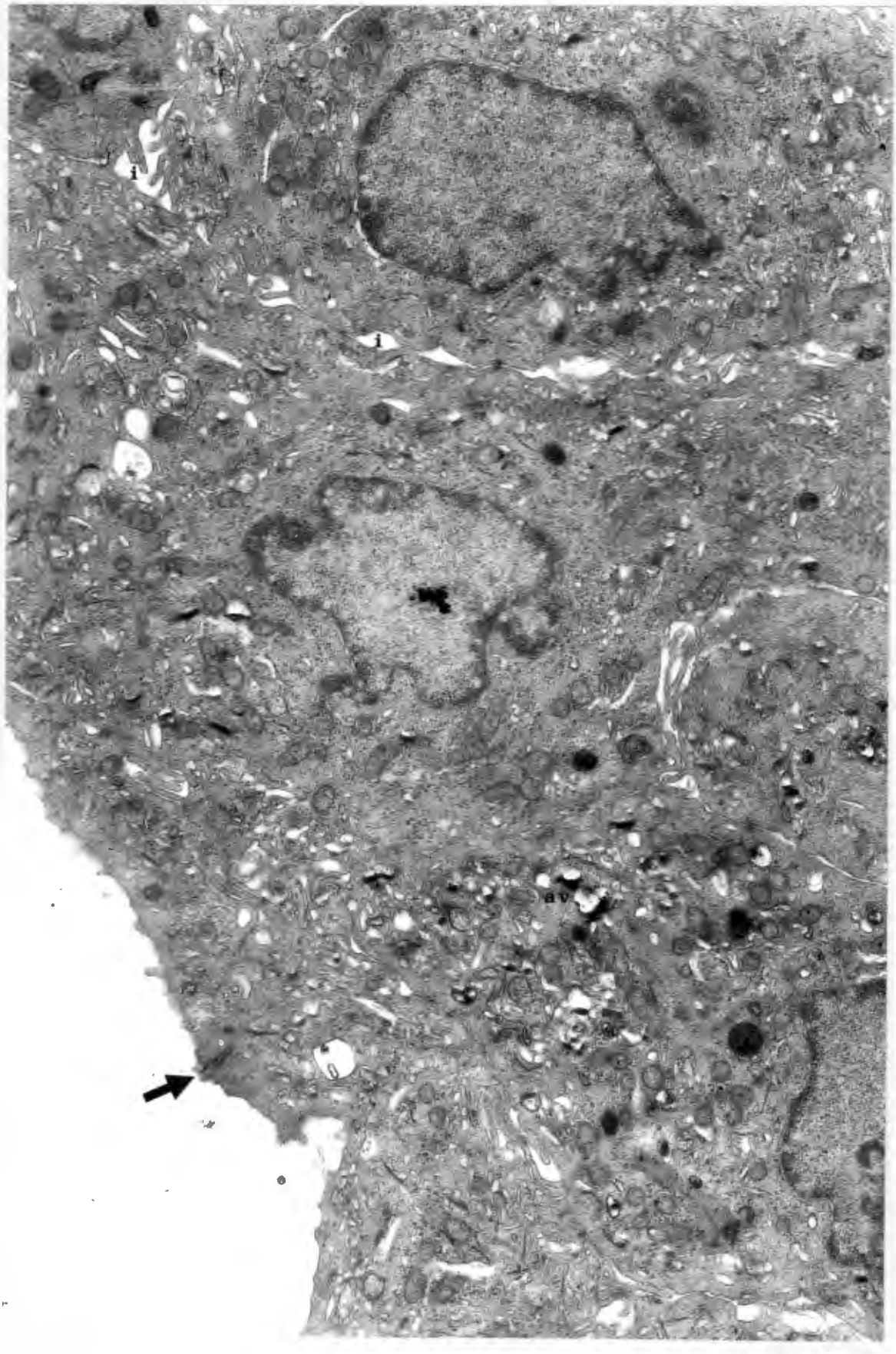


16

Figure 17. (X 20,000). The full thickness of the epithelium is composed of cells showing cytoplasmic and nuclear appearances similar to the intermediate cell layer. There is slight widening of the intercellular spaces (i). Autophagic vacuoles (av) are present in areas. The surface has lost its angular ridges and shows the presence of rounded projections. A tripartite junction (arrow) can be seen between adjacent surface cells. All the nuclei are indented suggesting that no normal surface cells are present.


i
i
av

Figures 18 & 19. Two stages of development of micro-papillae. Fig. 18 shows the initial outward folding of the epithelium into the bladder lumen. (H&E X 450). Fig. 19 shows three, or possibly four, micropapillae covered by the full thickness of the epithelium. There is a normal variation of cell type from the base to the surface. The "double" lesion at the one end shows an indistinct nodular inferior margin. (H&E X 450).

Figures 20 & 21. Two stages in the development of nodular hyperplasia. Fig. 20 shows an exaggeration of the appearance seen in figs. 10 & 11 with a suggestion that the undersurface is bulging down slightly into the underlying bladder wall. (H&E X 450). Fig. 21 shows the appearance of nodular hyperplasia. The nodules are delineated laterally by capillaries. Note that in this level, one of the capillaries reaches up to within three cell layers of the surface. (H&E X 450).

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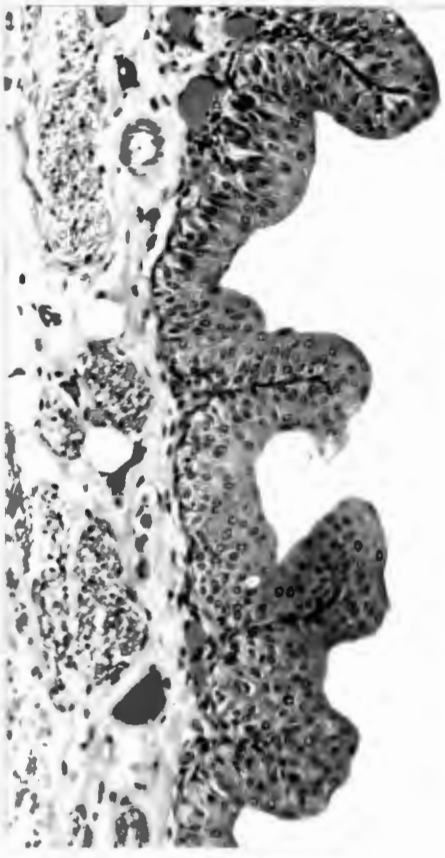

20

21

Figures 22, 23, 24, 25. Complex lesions showing endophytic and exophytic growth patterns in various proportions.

Figure 22. Mainly a flat lesion, it shows some pushing down of the epithelium into the underlying stroma, shown by compression of the connective tissue fibre, but also lifting of the epithelium into the bladder lumen. (H&E X 200).

Figure 23. This is a lesion, predominatly growing into the bladder lumen, showing features of nodular hyperplasia (arrows) but also showing a pseudo-acinar structure where the normal base to surface differentiation is present. Serial sections of this lesion showed that this pseudo-acinus resulted from infolding. At this deeper level the lesion had a more typical papillary pattern. (H&E X 200).

Figure 24. An exophytic lesion which at this level shows a large pseudo-acinar structure produced by infolding. (H&E X 200).

Figure 25. A papillary lesion showing some nodular down-growths at the base (arrow). (H&E X 75).

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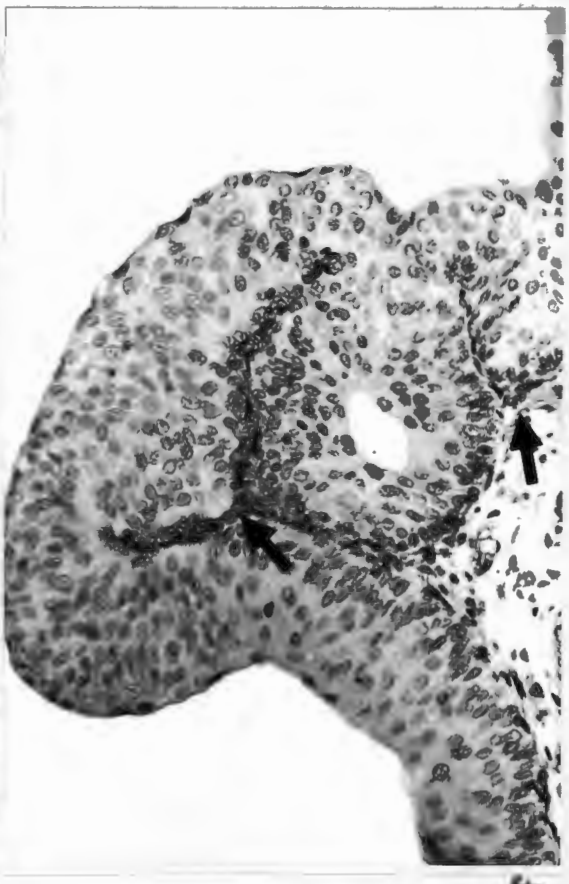

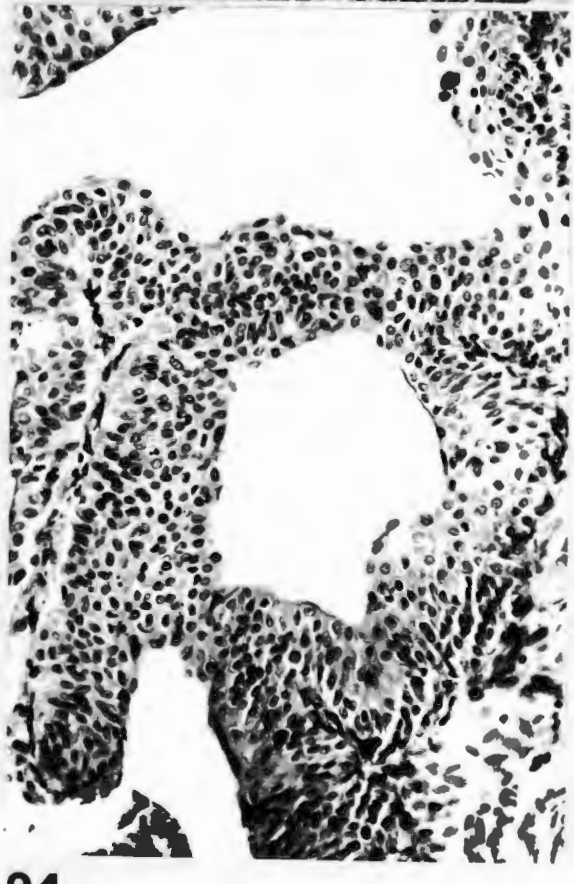

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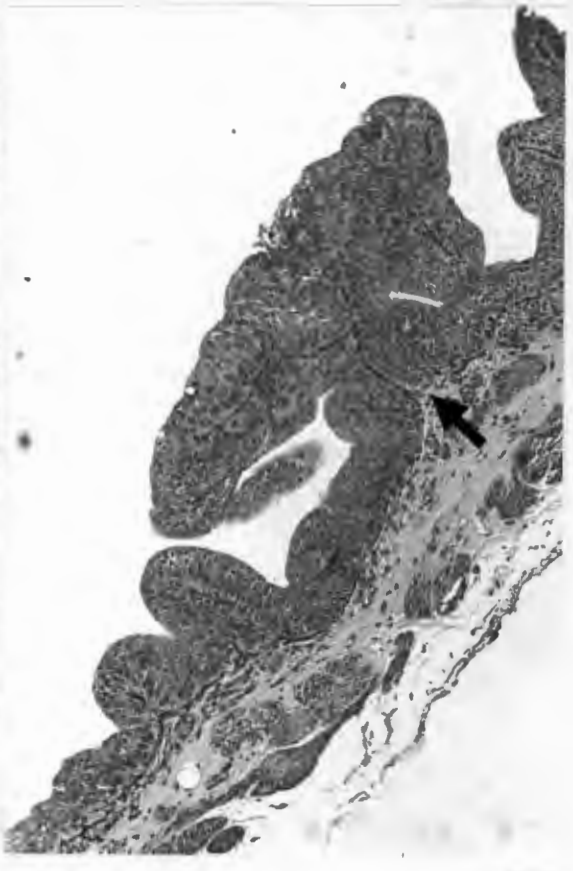
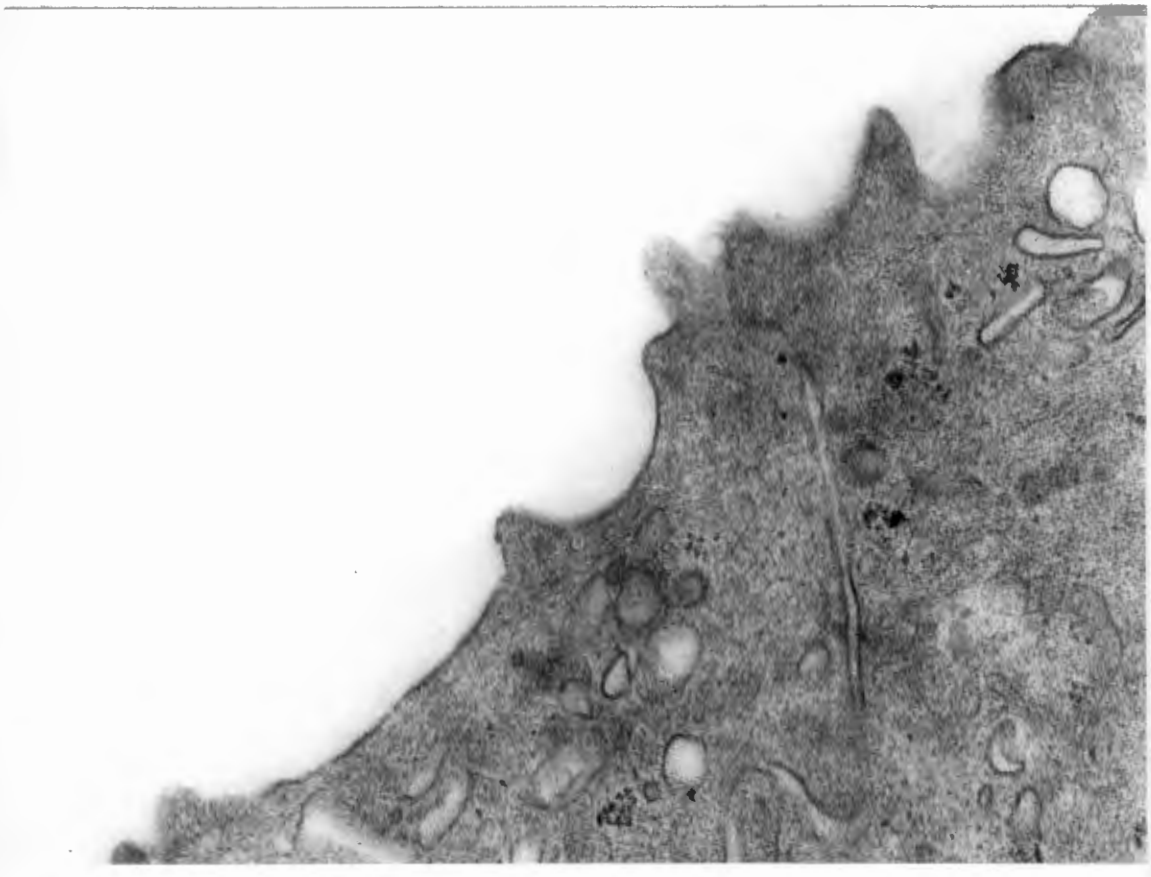
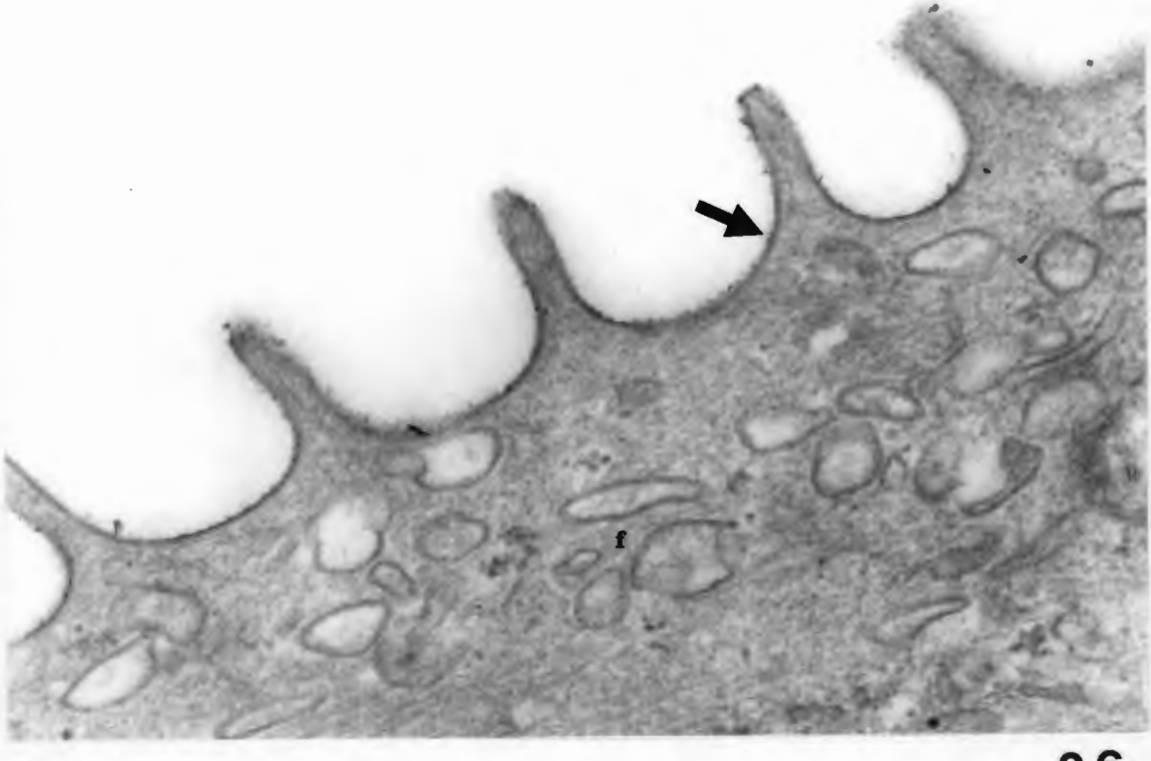


25

Figure 26. a & b. (X 10,000). Both these illustrations show the replacement of angular ridges by rounded projections. The surface membrane cannot be distinctly seen but short segments in b. (arrow) show that the asymmetry is still present. Fusiform vesicles (f) are present in both examples. Both these illustrations are taken from nodular tumours. The exaggerated projections shown in b. were not common.

26a





26b

Figure 27. (X 20,000). Papillary tumour showing mainly straight cell boundaries which are interdigitate only where three cells meet (arrow). A large telolysosome (tl) is present. Autophagic vacuoles (av) are scattered throughout the cells.

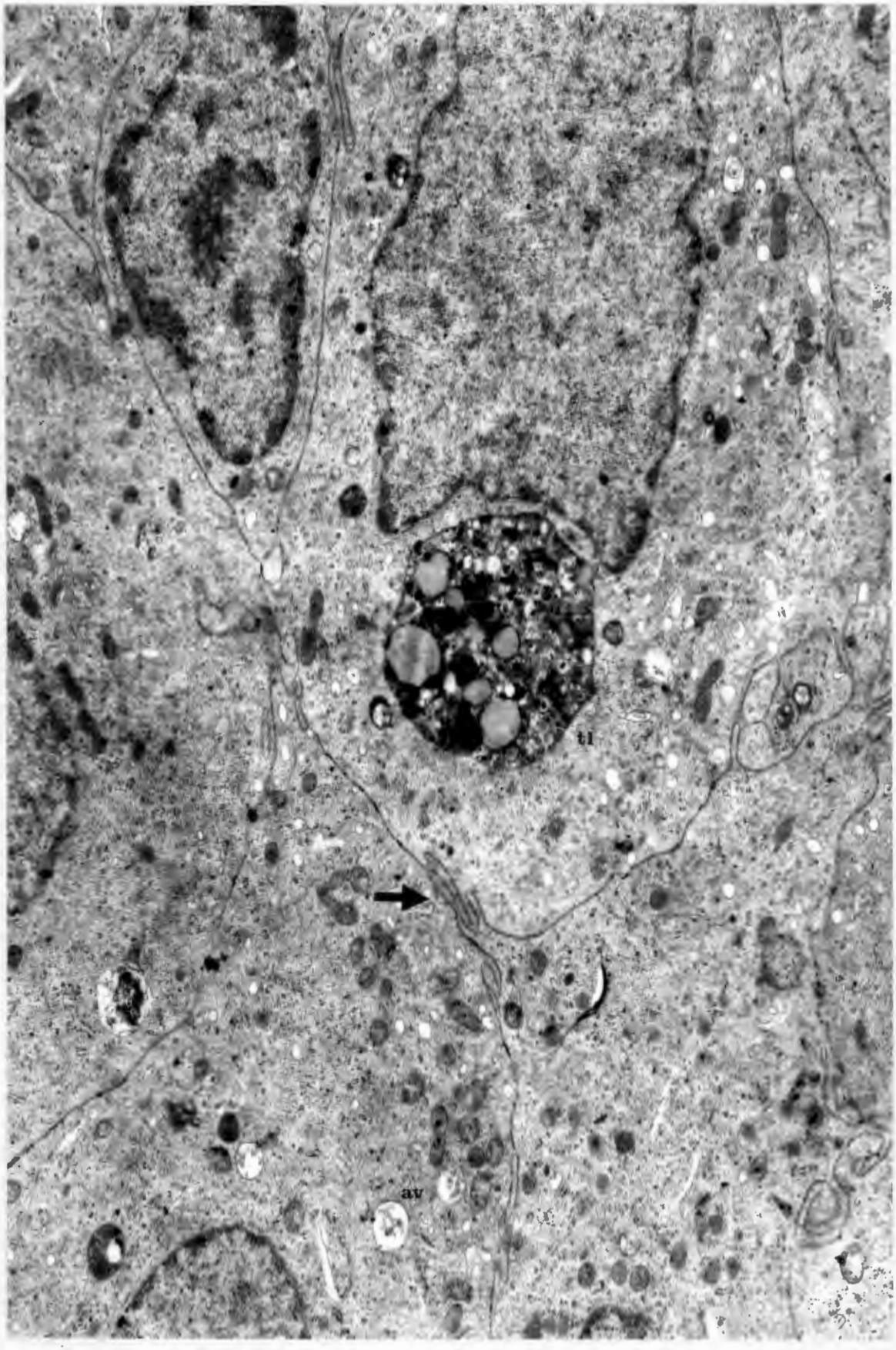
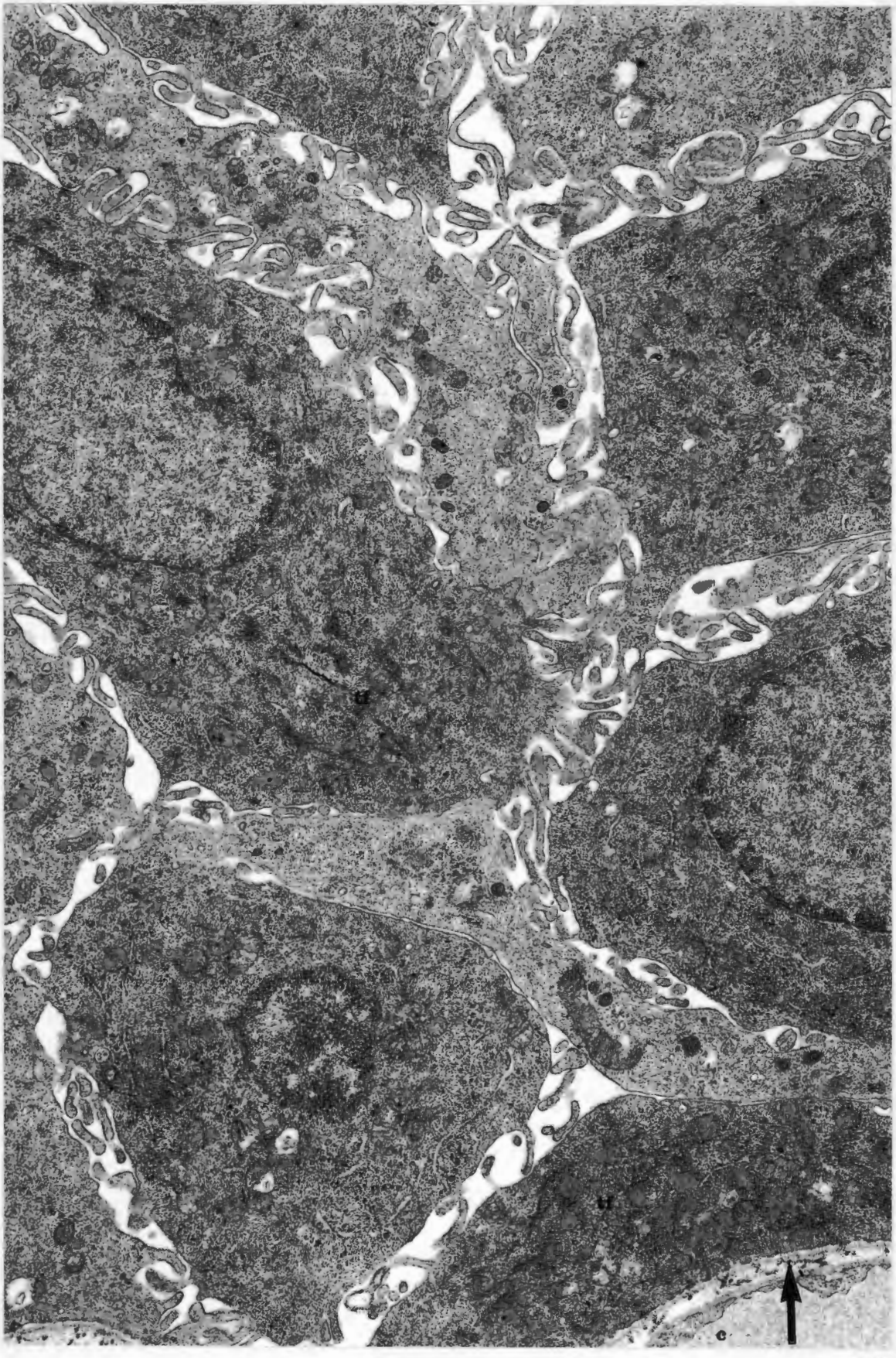

tl
av

Figure 28. (X 20,000). A nodular lesion showing separation of the cells by widening of the inter-cellular space. This space contains interdigitating cytoplasmic projections. At the lower right is the basal lamina (arrow) with capillary (c) below. Many of the cells contain prominent tonofilaments (tf).



28

Figure 29. (X 120,000). A higher magnification of the widened intercellular space seen in nodular lesions. The interdigitating cytoplasmic projections are well seen. A large mitochondrion (m) and several smaller mitochondria are present. Desmosomes are absent.

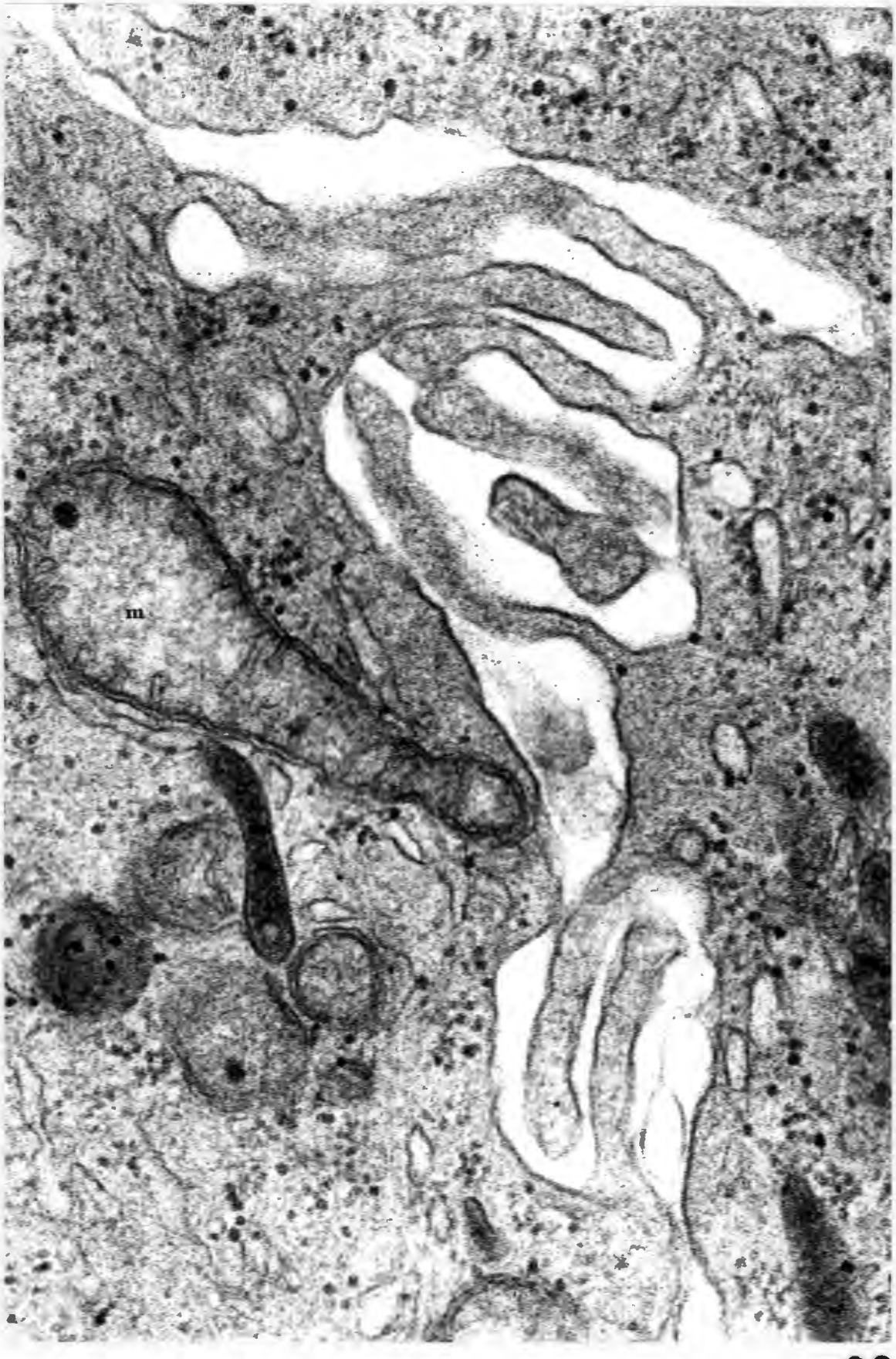

m

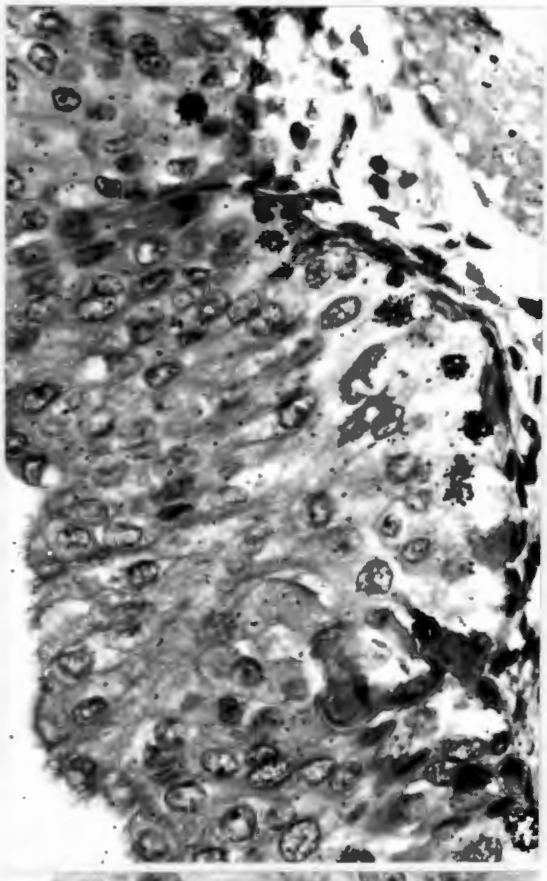
Figure 30. Autoradiograph of early nodular hyperplasia showing labelled cells confined to the basal layer (H&E X 450).

Figure 31. Telophase in the base of a micropapillary lesion showing separation parallel to the basal lamina (H&E X approx. 600).

Figure 32. Telophase in non-papillary hyperplastic epithelium showing separation at right angles to the basement membrane (H&E x approx. 600).

Figure 33. Autoradiograph of papillary lesions. Only a few labelled cells are present most of which are not in the lesion itself. At the base of the fibro-vascular core (arrow) is a labelled stromal cell (H&E X 200).

30



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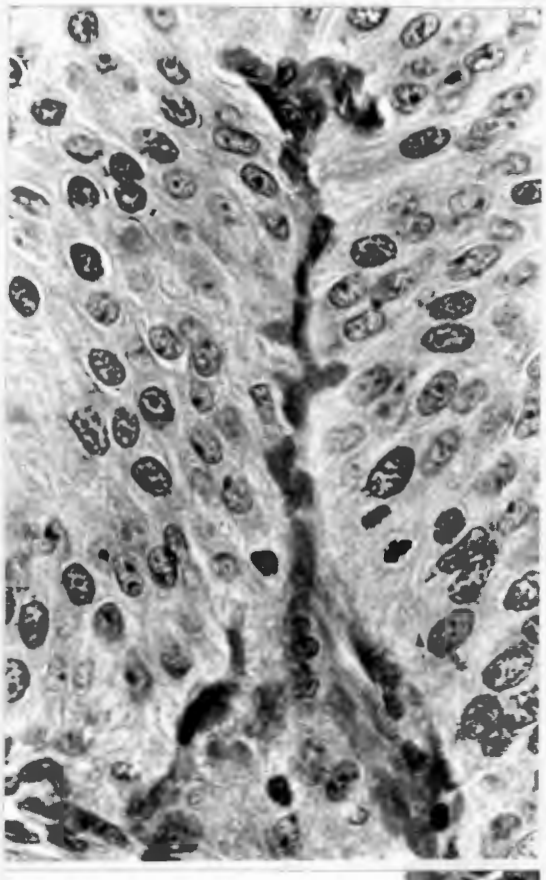

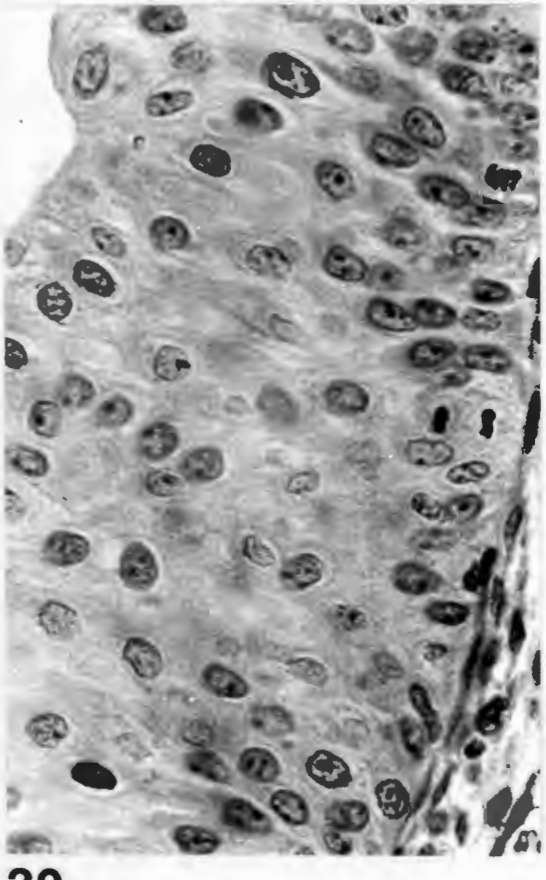

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Figures 34 & 35. Two examples of papillary tumours. A pseudo-acinar pattern was commonly found in these lesions. Note that base to surface differentiation is present. (H&E Fig. 34 X 100, Fig. 35 x 100).

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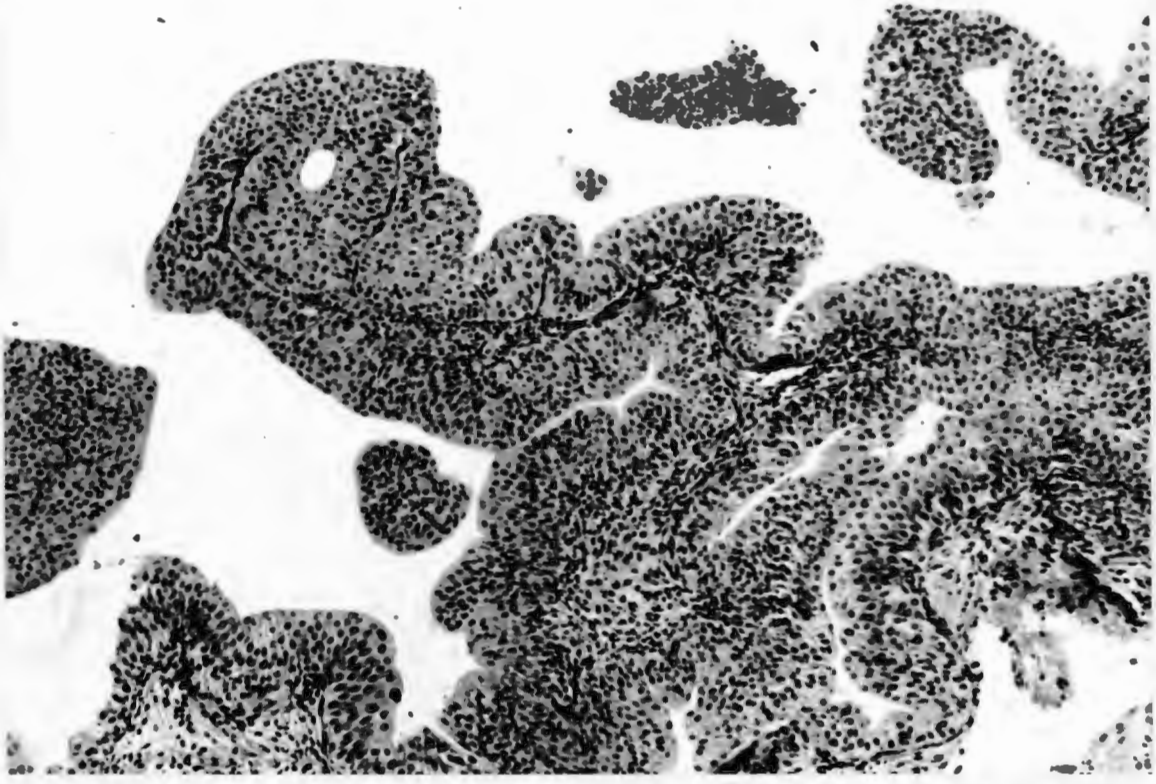
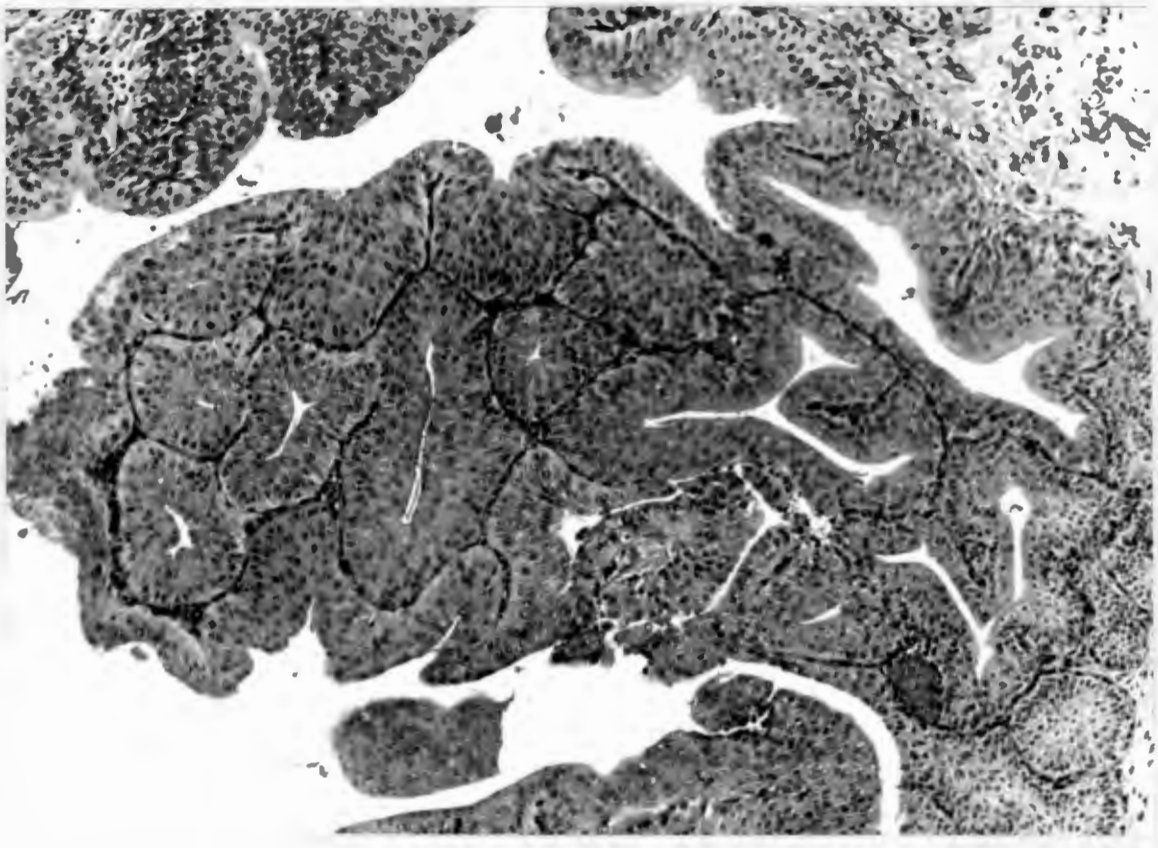
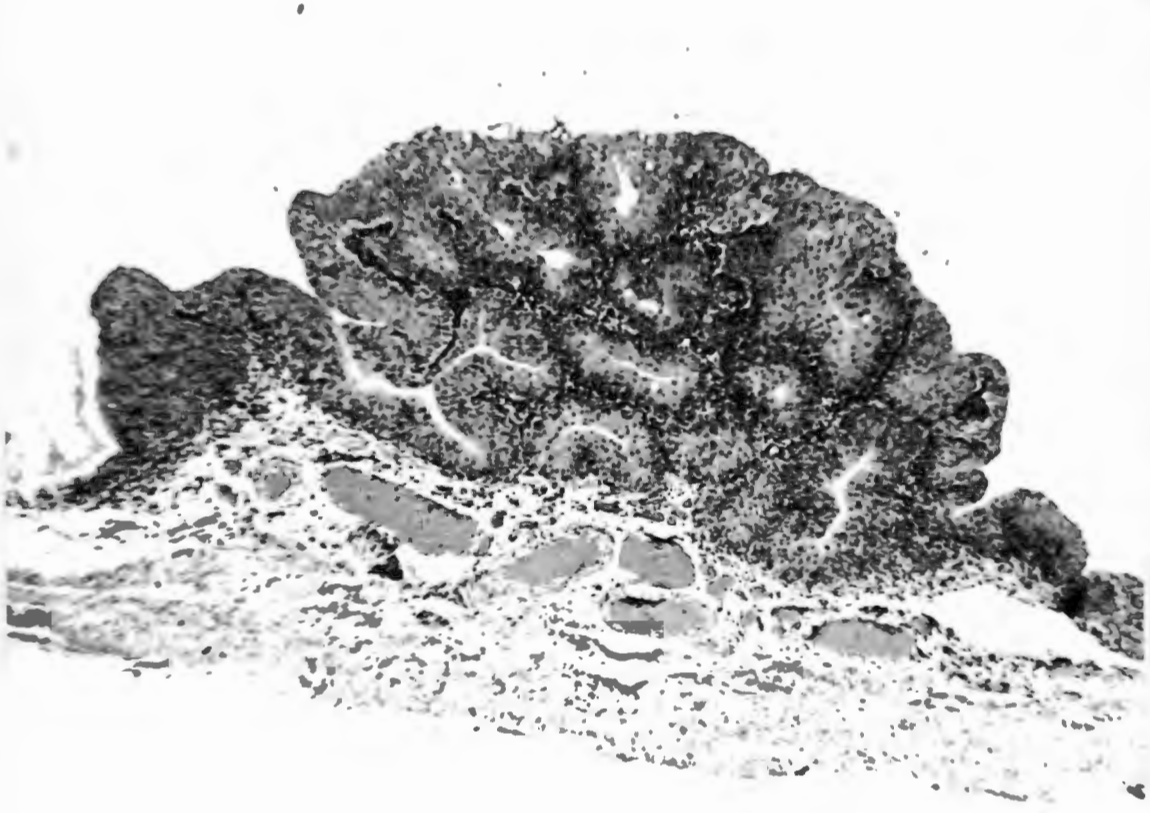


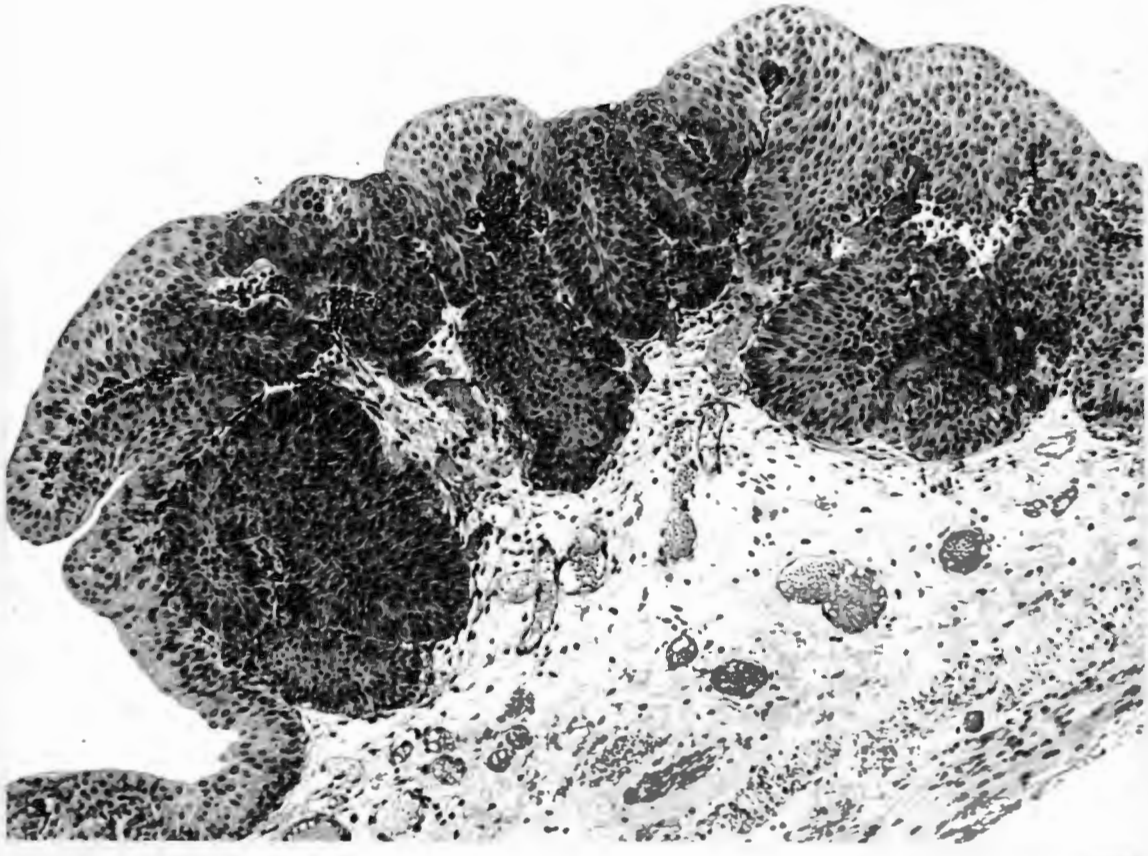


35

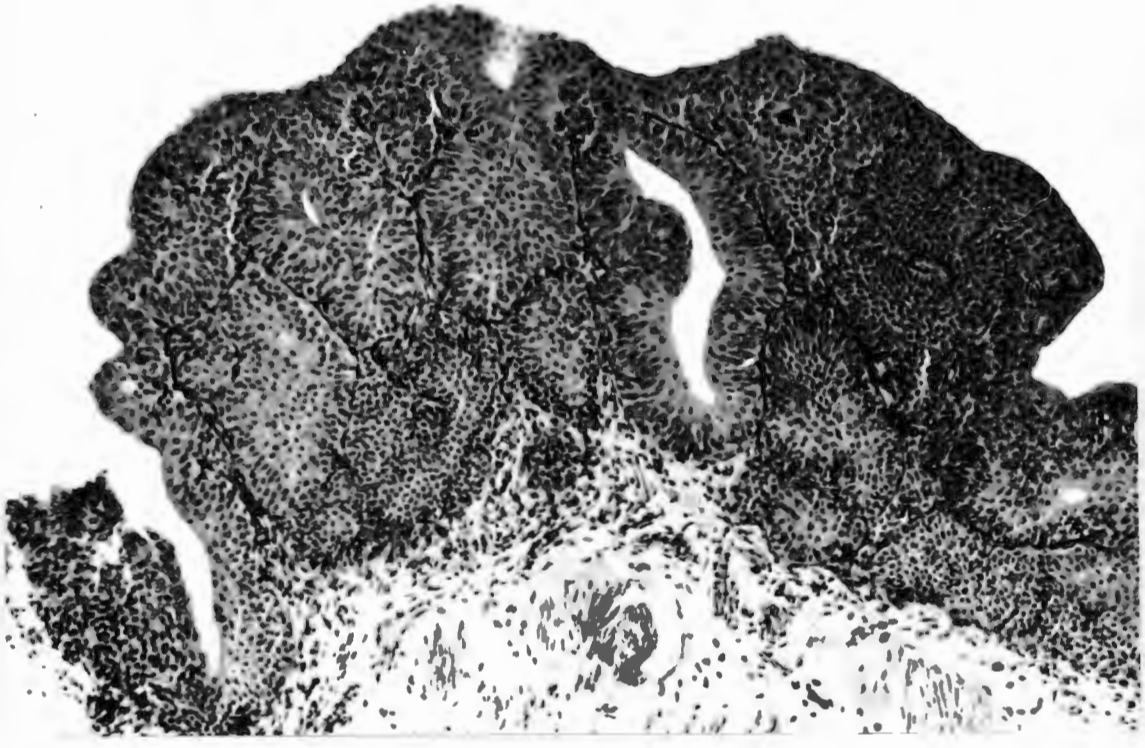
Figure 36. A broad based papillary tumour. A pseudo-acinar pattern is dominant. Note the dilated blood vessels at the base. (H&E X 64).

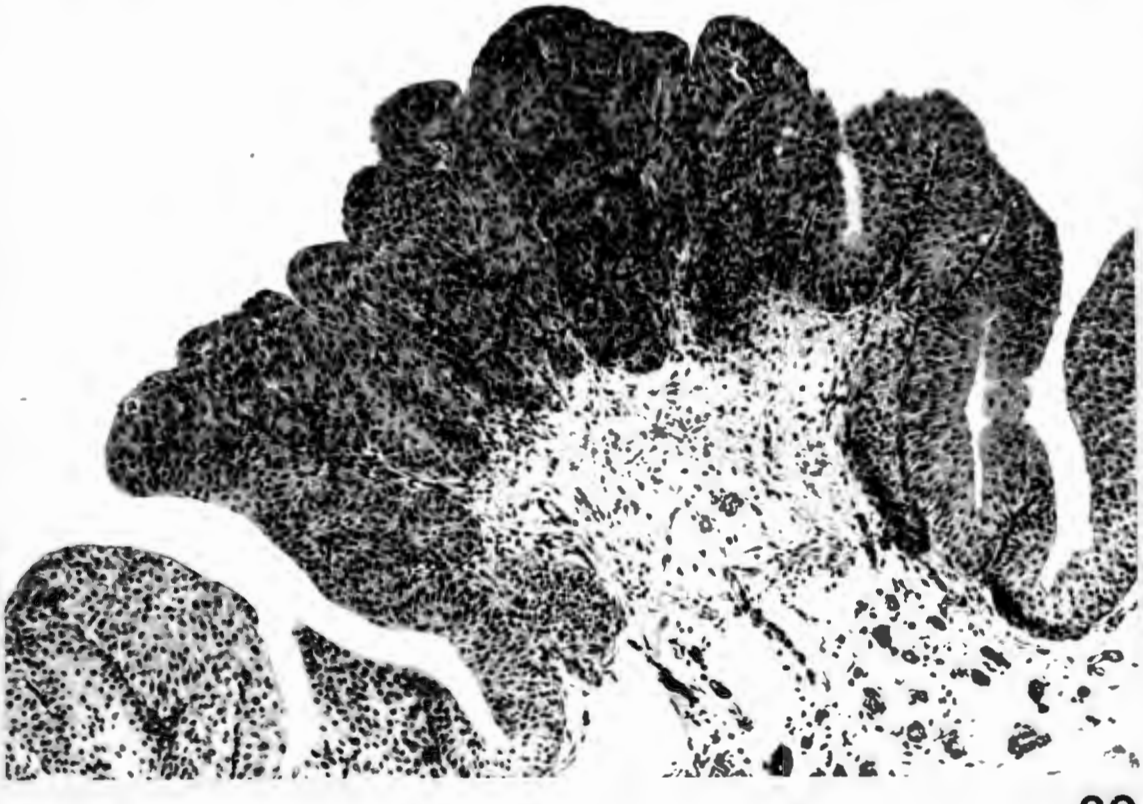
Figure 37. A polypoid nodular tumour. The central core is composed of vascular connective tissue. The epithelium shows an exaggeration of nodular hyperplasia. In a central situation the full thickness of the epithelium is involved but elsewhere the surface of the epithelium has a differentiated appearance and the hyperplastic epithelium appears to originate from the basal area. (H&E X 100).





Figures 38 & 39. Combined tumours. Fig. 38 shows a pseudo-acinar structure within a tumour which otherwise has a nodular configuration (H&E X 100). Fig. 39 shows a nodular lesion with papillary formations at the periphery. (H&E X 100).





39

Figure 40. Autoradiograph. Despite the low magnification of this illustration, which has been used to include the whole lesion, it is possible to see that the large number of labelled cells are situated in the basal layers. (H&E X 75).

Figure 41. Autoradiograph. This is the edge of a nodular tumour. While most of the labelled cells are in a basal situation a single superficial cell is labelled (arrow) and others away from the basal layer are also labelled. The basal layer is not only that on the inferior border of the tumour but is also that which abuts on the fibrovascular, capillary containing, cores within the tumour. (H&E X 200).

Figures 42 & 43. These are the two halves of the bladder of a test animal sacrificed at week 35. Many tumours are shown. The largest of these are situated in the fundus. In this terminal stage of the experiment tumours of various sizes were seen also situated on the lateral walls.

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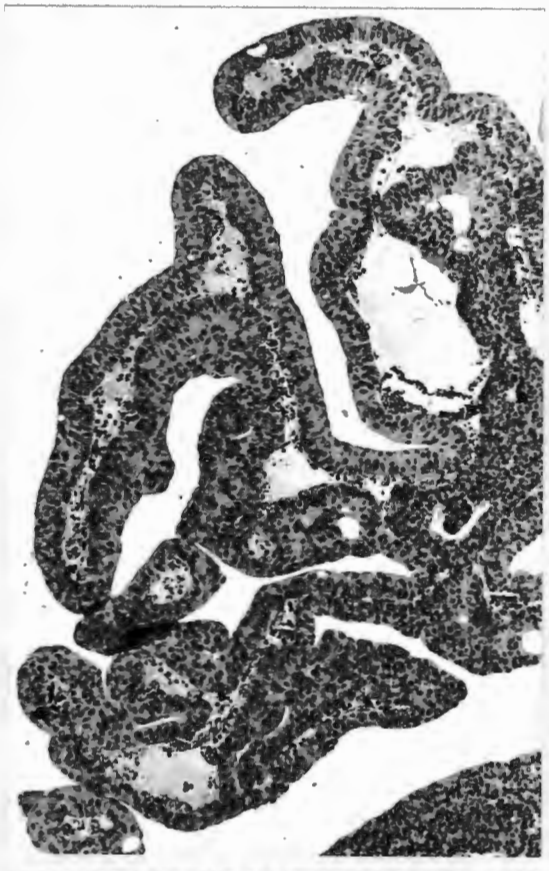
Figure 44. A well differentiated papillary tumour from a Test animal sacrificed at week 35.
(Alcian blue X 75).

Figure 45. Narrow bands of epithelial cells extending down from the surface into the underlying stroma of the tumour. (H&E X 75).

Figures 46 & 47. Higher magnifications of the narrow bands of epithelial cells to illustrate the apparent differentiation from the periphery to the centre.
(H&E fig. 46 X 150; Fig. 47 X 600).

44



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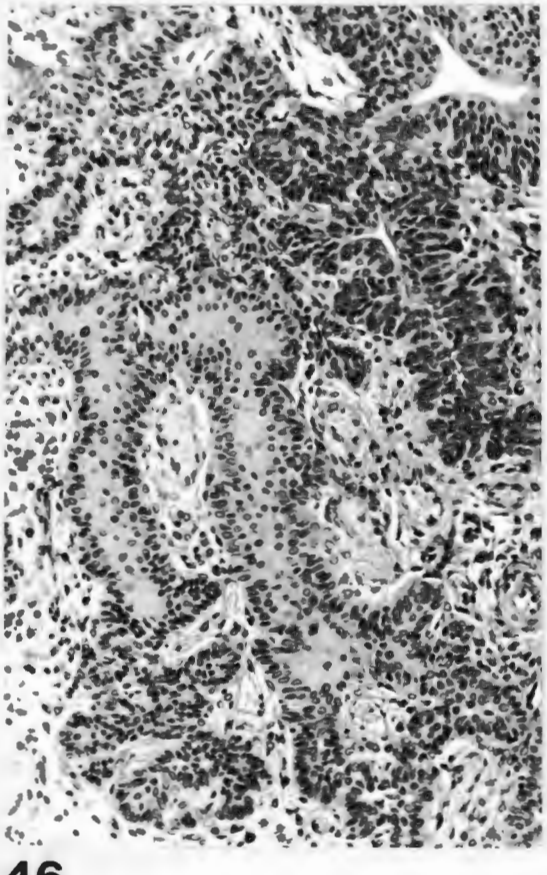

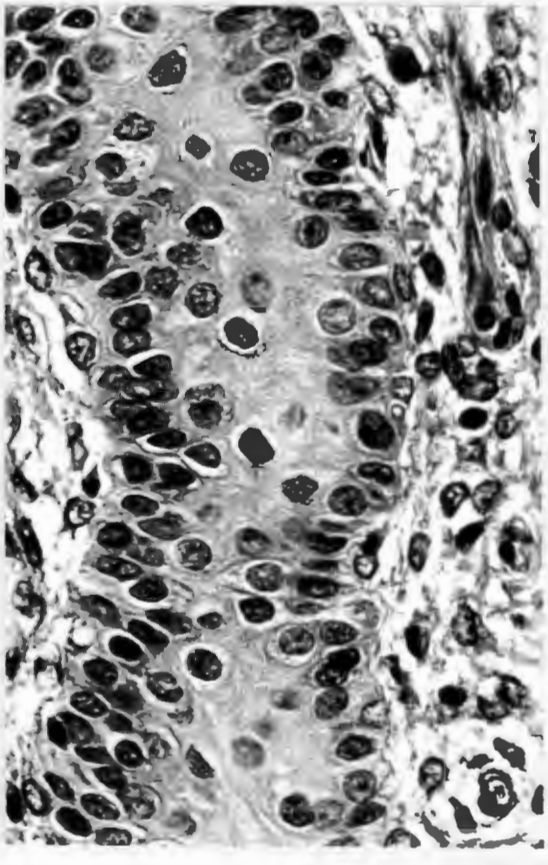

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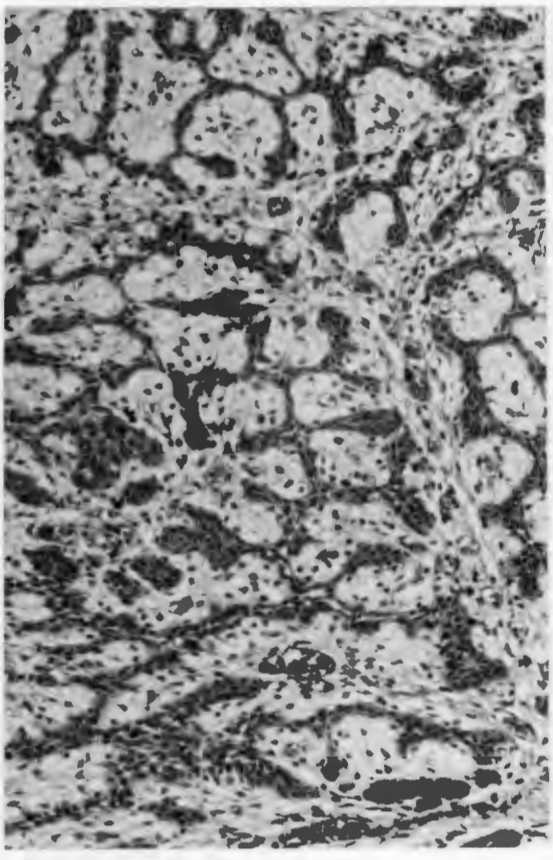
Figures 48 & 49. Two magnifications to illustrate the fully developed filigree pattern. The anastomising bands of cells were, in areas only two cells thick. The connective tissue appears loose and oedematous. (H&E fig. 48 X 150; fig. 49 X 600).

Figure 50. The bands of epithelial cells extending down from the surface were often luminated and continuous with the bladder lumen. (Reticulin X 75).

Figure 51. Autoradiograph of filigree tumour. Most of the labelled cells are in the epithelial bands. Three labelled endothelial cells can be seen in the same vessel. (H&E X 450).

48



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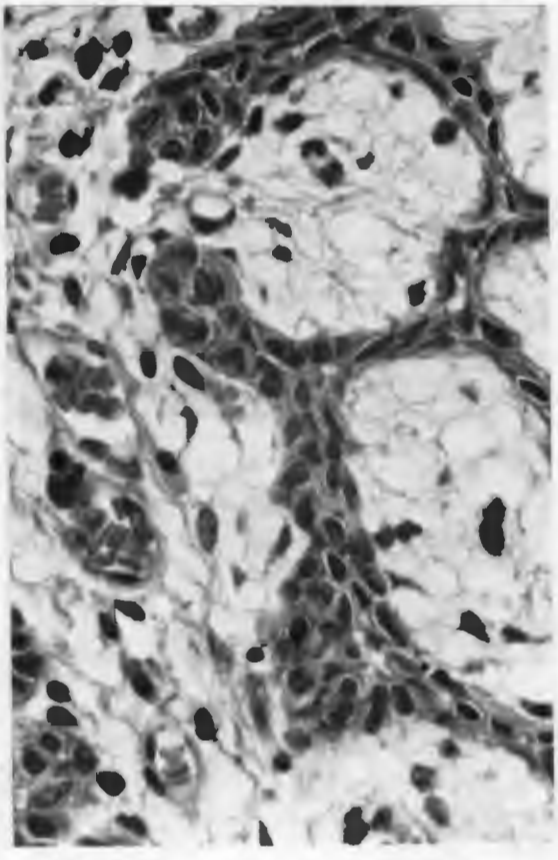

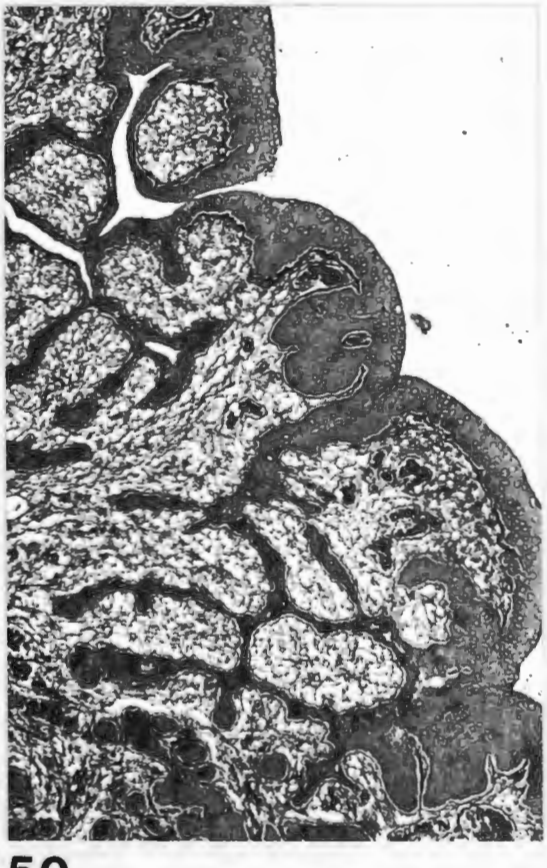

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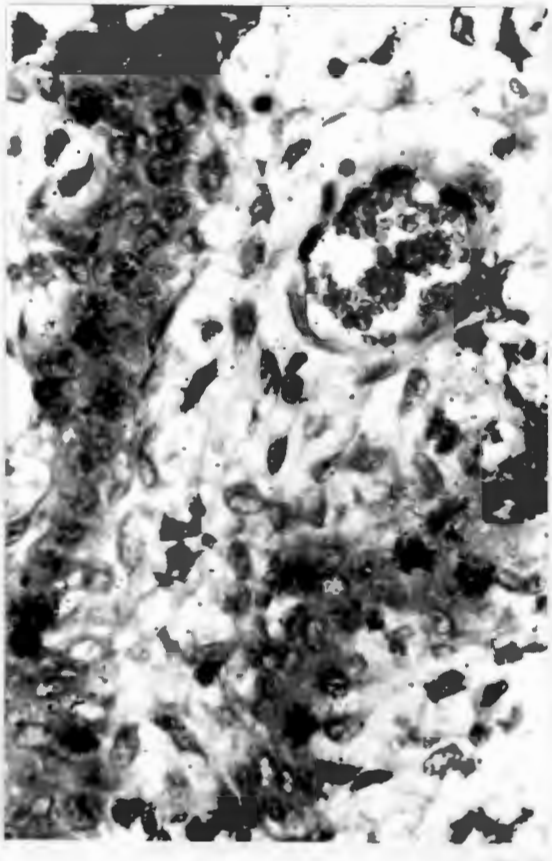


51

Figure 52. Solid epithelial tumour. The epithelial cells are broken up into small groups by a fine reticulin network but there is no more connective tissue than this. (H&E X 75).

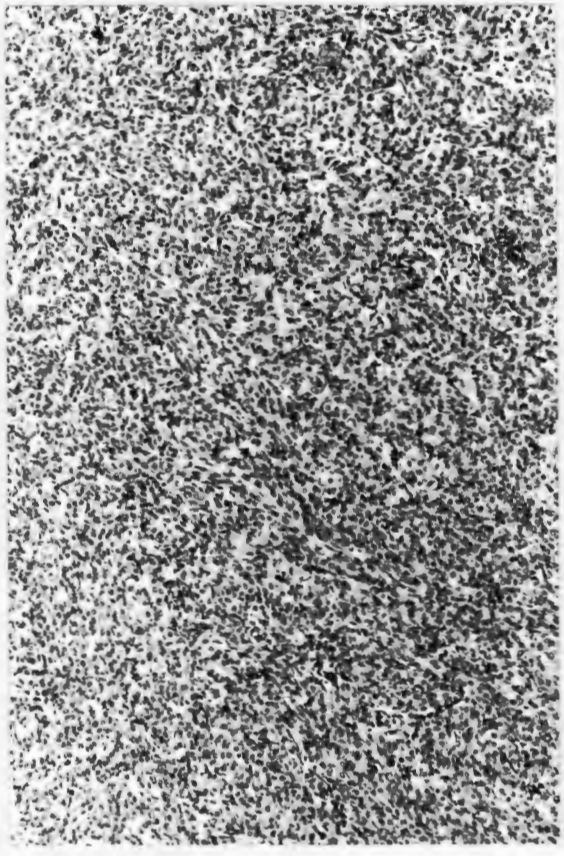
Figure 53. Pseudo-acinar structures occurring within solid epithelial tumours. (H&E X 200).

Figure 54. Autoradiograph of solid epithelial tumour showing large number of labelled cells not confined to the basal areas. (H&E X 600).

Figure 55. a. Smooth endoplasmic reticulum which was increased in some cells in the larger tumours. A portion of mitochondrion is present. (X 120,000).

b. Laminated bodies which appeared to be phospholipd bodies. (X 120,000).

52



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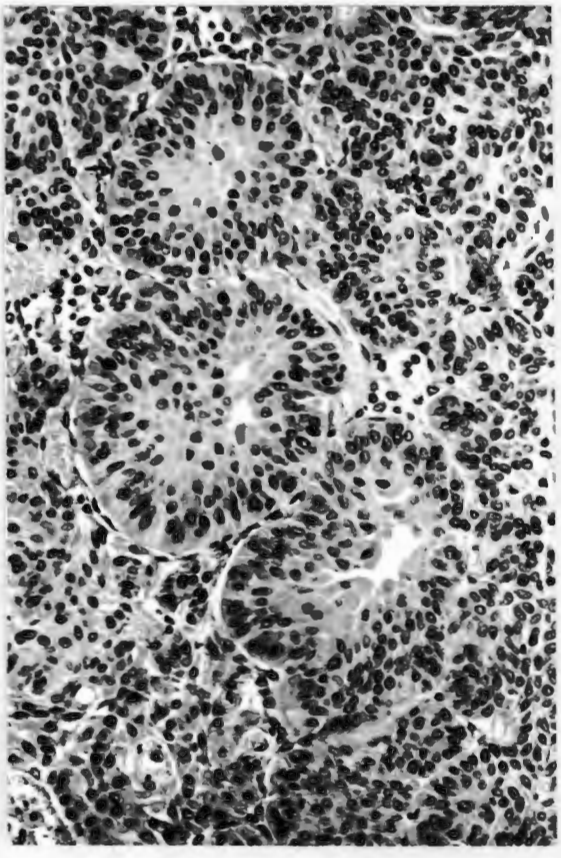

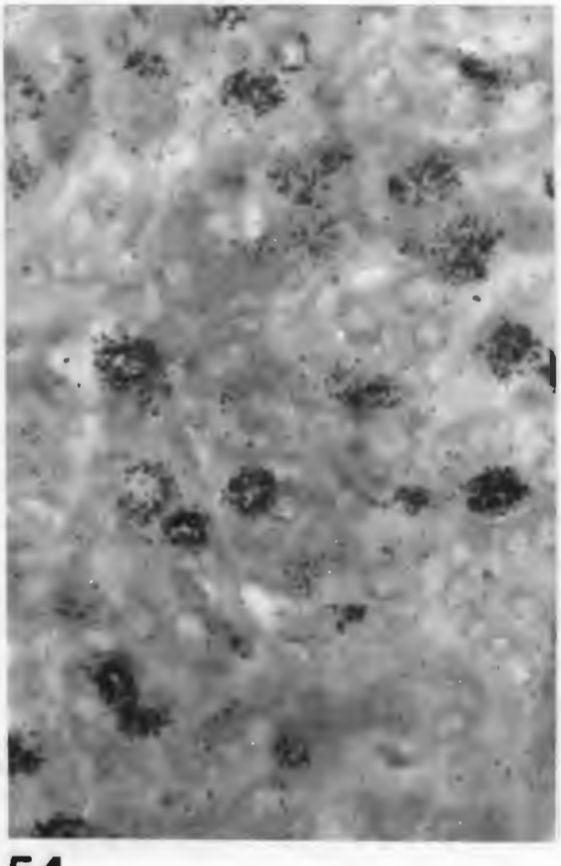

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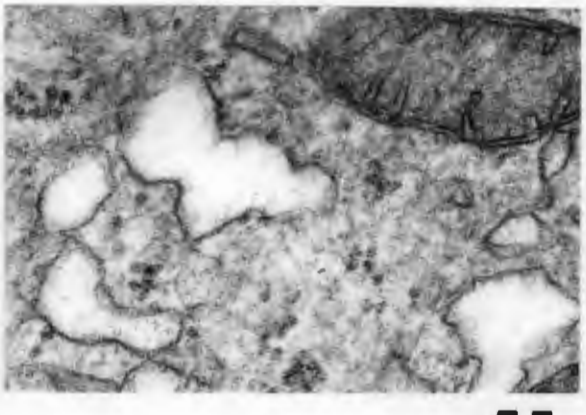

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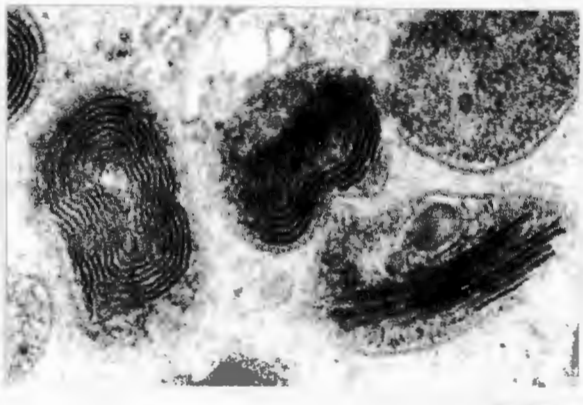


55b

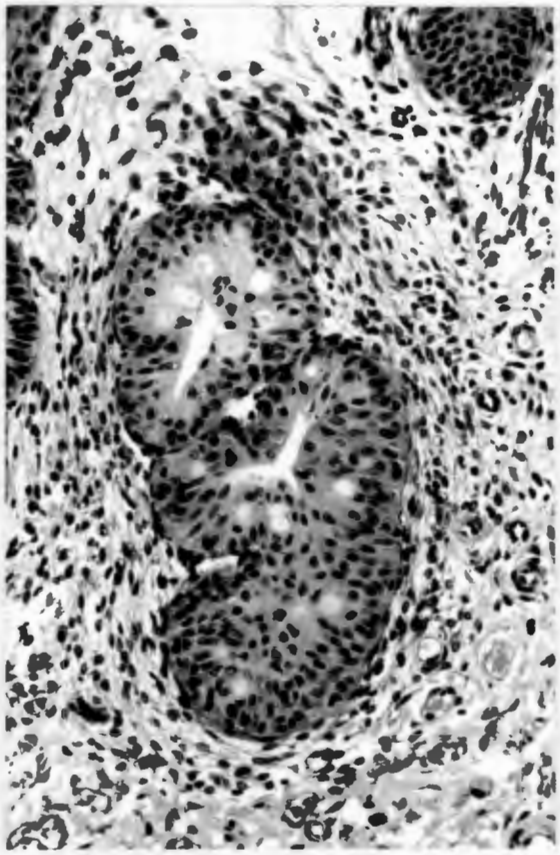
Figure 56. Isolated islands of cells lying within the connective tissue stroma of a papillary tumour. The centre is luminated and there is a differentiation from the periphery to the centre. There is only minimal compression effect on the surrounding connective tissue but the periphery of the cell nest is smooth. (H&E X 200).

Figure 57. Sharp spurs of epithelial cells placed between nodules of hyperplastic epithelium. This sharp spur appears to be splitting rather than compressing the connective tissue. (H&E X 600).

Figure 58. Islands of cells lying in the base of a nodular tumour. There is no variation in cell type from the periphery to the centre. The perimeter of the islands are not so well delineated as that in fig. 56. (H&E X 600).

Figure 59. Tumour Cells apparently invading a thin walled blood vessel.

56



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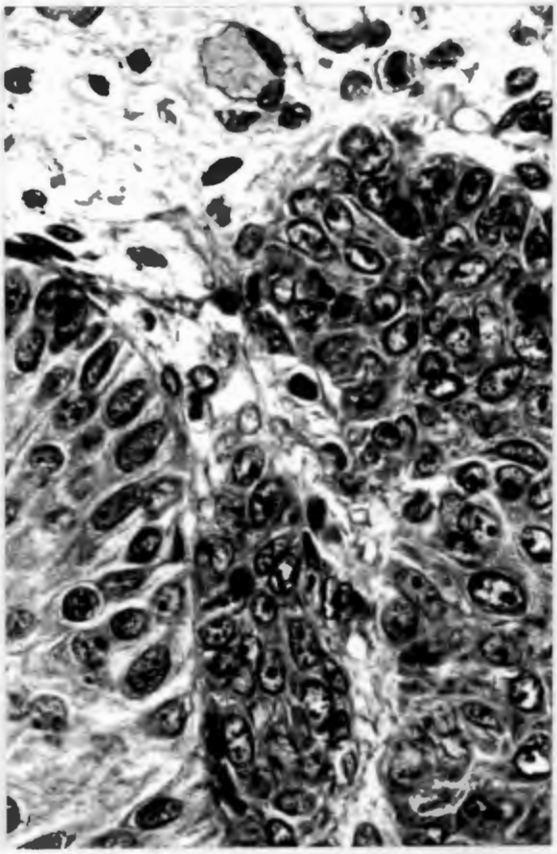

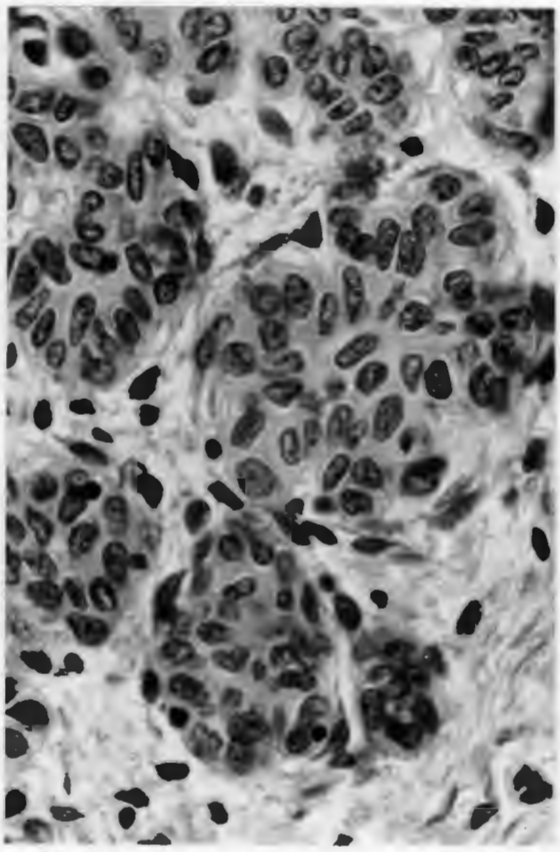

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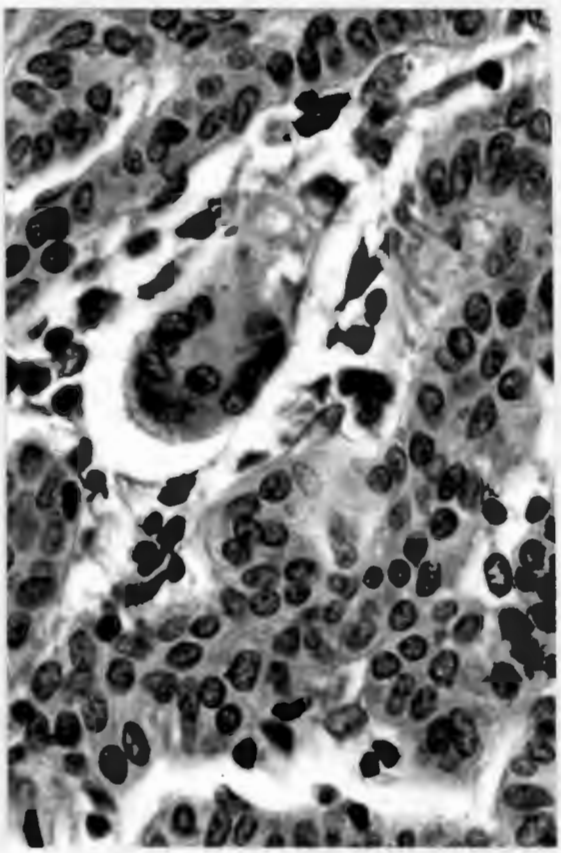

59

Figures 60 & 61. Two magnifications of carcinoma cells infiltrating the bladder wall. The cells are small and can be seen insinuating themselves between the connective tissue fibres. (H&E fig. 60 x 200; fig. 61 X 450).

Figures 62 & 63. Two magnifications of carcinoma cells infiltrating the bladder wall. The cells are large with abundant, almost granular cytoplasm. Although, at low magnifications, they appear to be invading en masse some individual cells are surrounded by reticulin. (H&E fig. 62 X 150; fig. 63 X 600).

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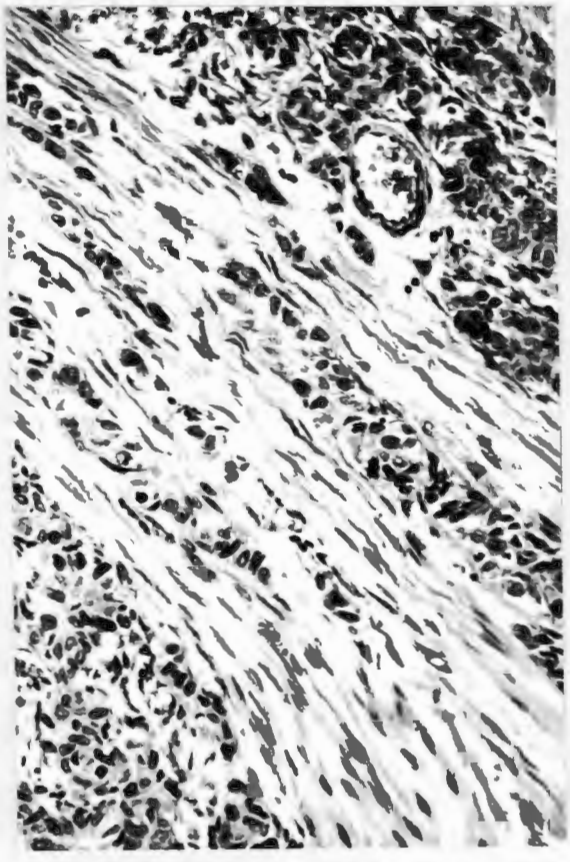

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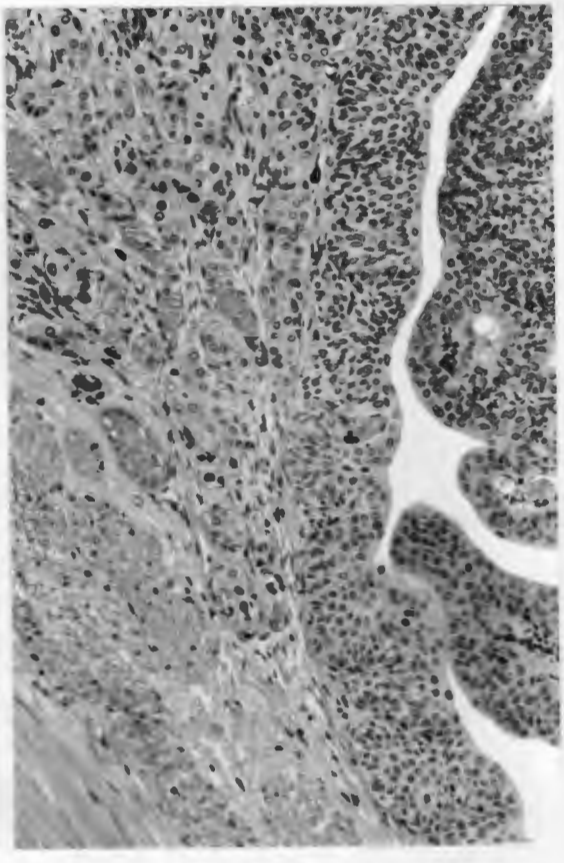

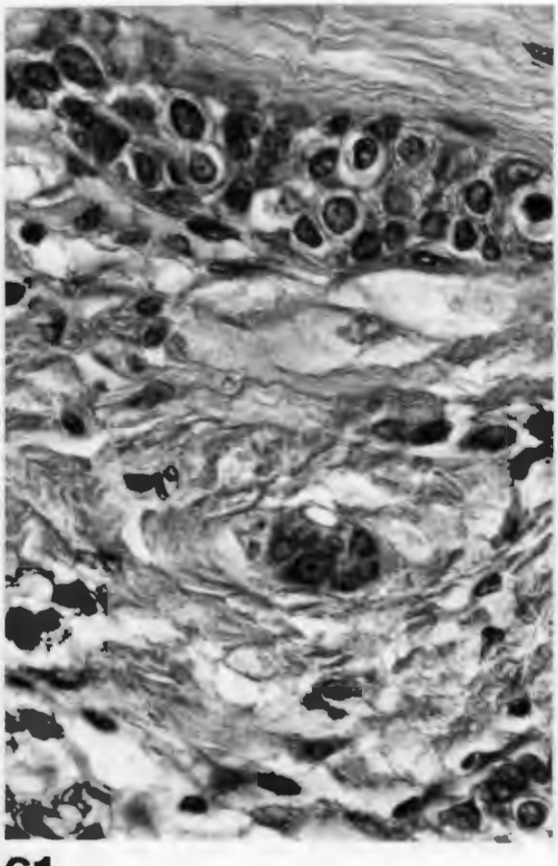

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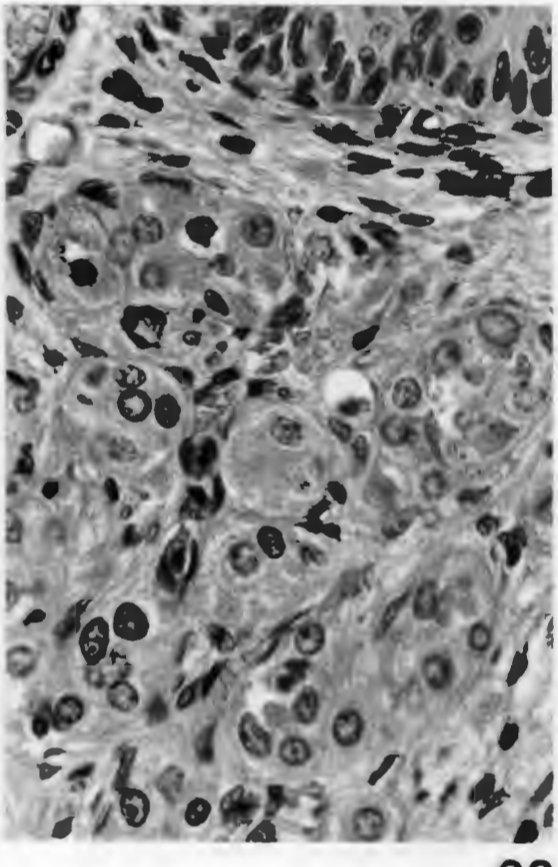


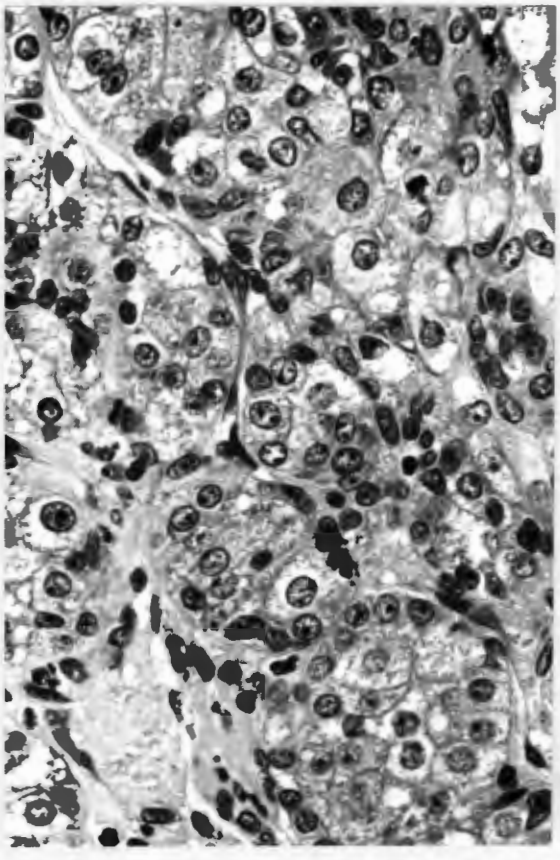
63

Figure 64. The cells of the metastatic tumour deposit found in the groin. These cells are similar to those in fig. 63. The lesion illustrated in fig. 63 was present in the bladder of the same animal in which this metastasis was found. (H&E X 450).

Figure 65. Autoradiograph of this metastatic tumour showing large numbers of labelled cells. (H&E X 200).

Figures 66 & 67. Early squamous metaplasia. A portion of the transitional epithelium is replaced by keratinising squamous epithelium. Fig. 66 is a small nodular lesion (X 200) and fig. 67 is papillary (X 200) (H&E).

64



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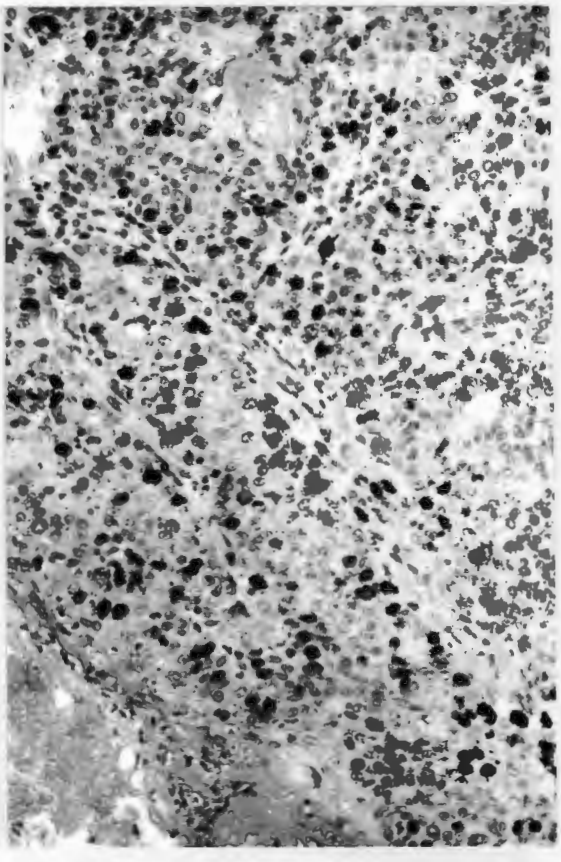

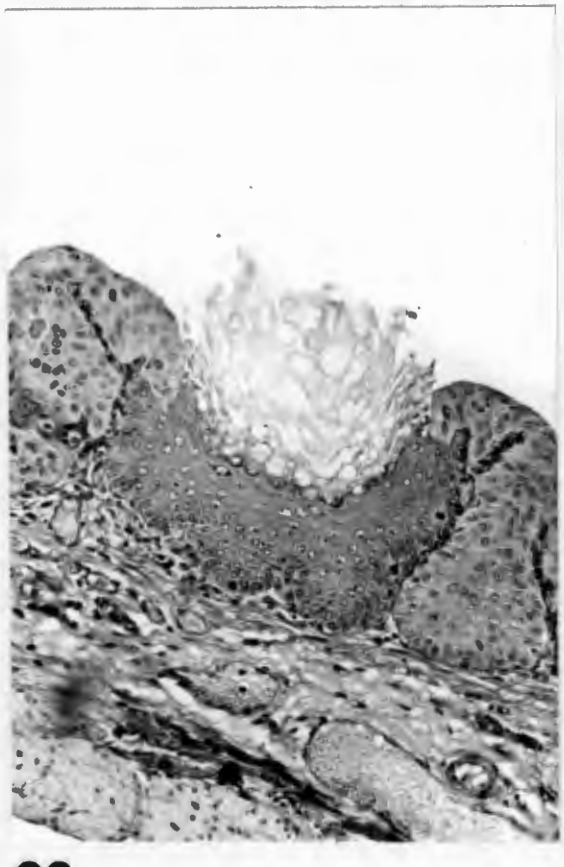

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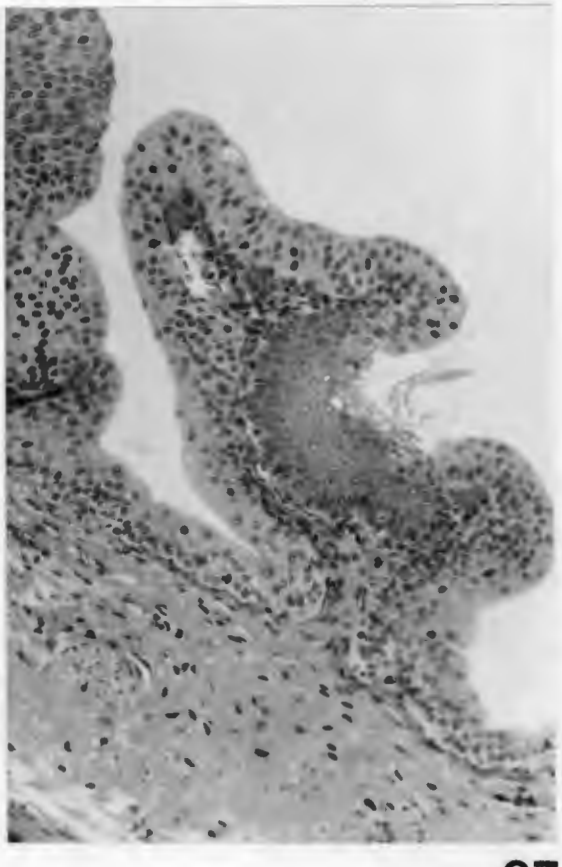


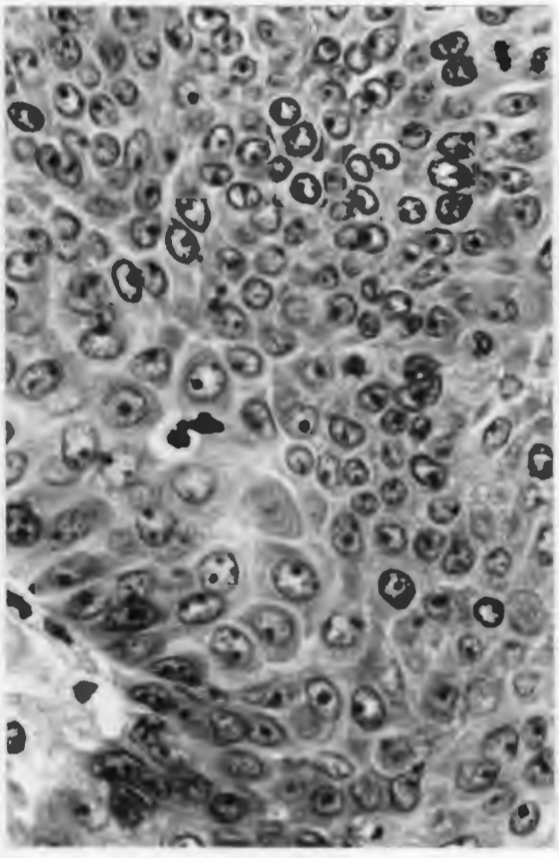
67

Figure 68. The squamoid change seen in nodular lesions. The cells have an angular appearance and are separated from each other by a widened intercellular space. (H&E X 600).

Figure 69. A large tumour showing an area of keratinising squamous epithelium (arrow) (H&E X 60).

Figures 70 & 71. Autoradiographs, at two different magnifications, of a keratinising squamous tumour. The tumour illustrated was connected to the bladder wall by a narrow stalk. The squamous epithelium shows an orderly maturation through a prickle cell layer to the surface. Labelled cells are confined to the basal layer.
(H&E fig. 70 X 75; fig. 71 X 200).

68



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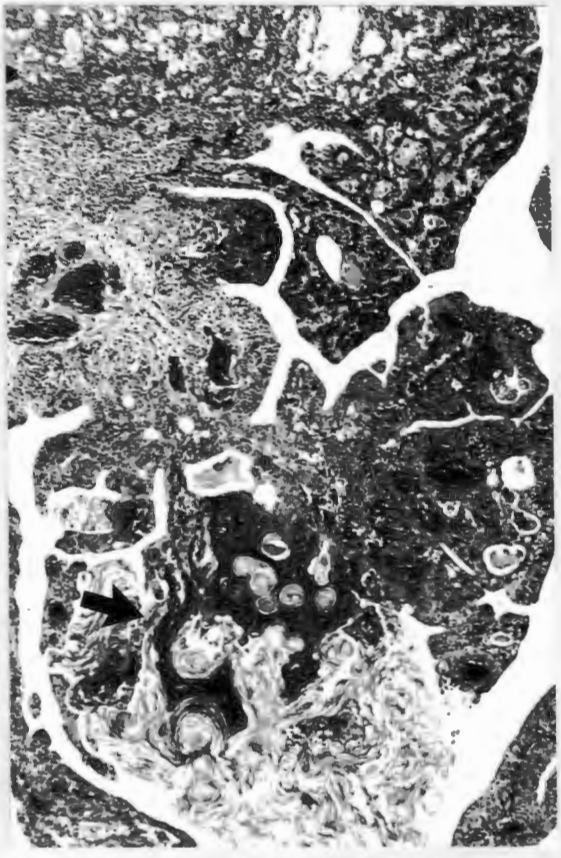

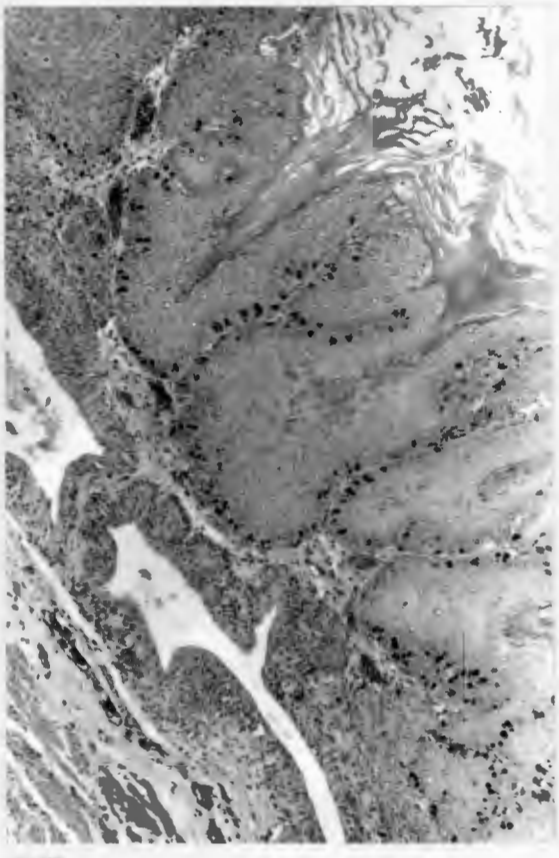

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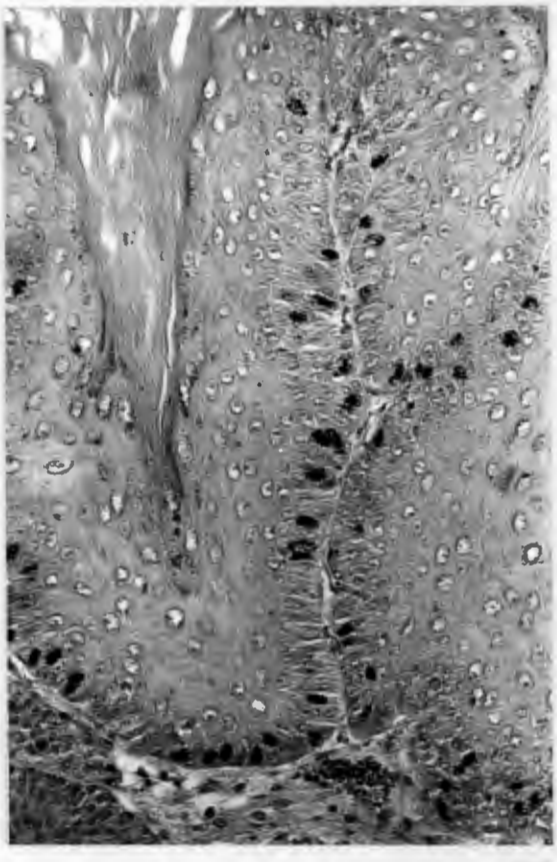

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Figure 72. (X 80,000). An area in a nodular lesion showing increased intercellular space with prominent tonofilaments (tf) some of which are running into desmosomes (dm). This appearance suggests that these areas may represent early squamous metaplasia.

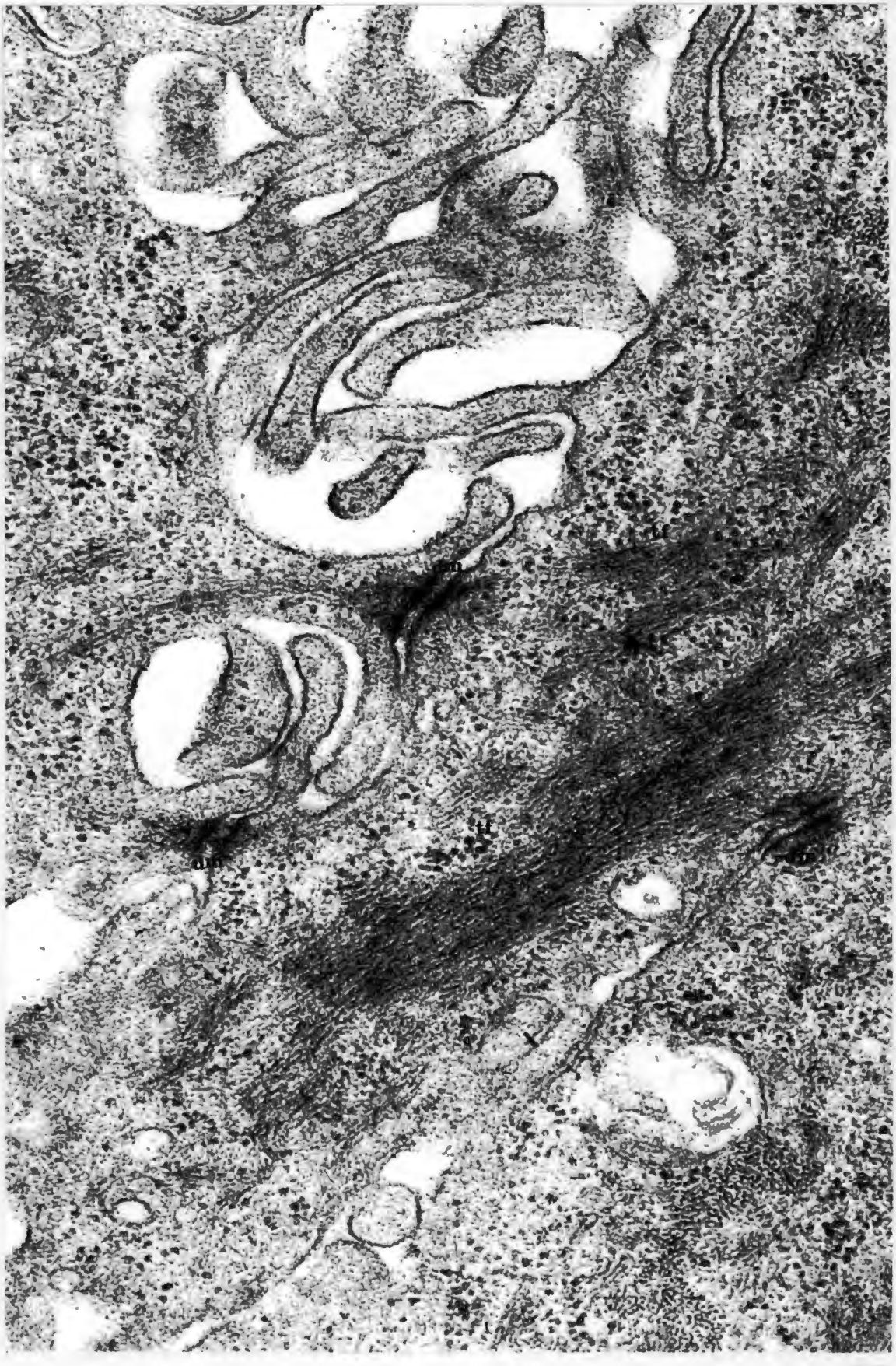

Figure 73. (X 60,000). Basal cells in a large tumour at the terminal stages of the experiment. The basal lamina (arrow) is shown at the bottom of the illustration. The basal cells contain prominent tonofilaments (tf). Desmosomes (dm) are poorly seen. Half desmosomes (hd) are seen on the basal cells joining these cells and the basal lamina. Mitochondria (m) are present.

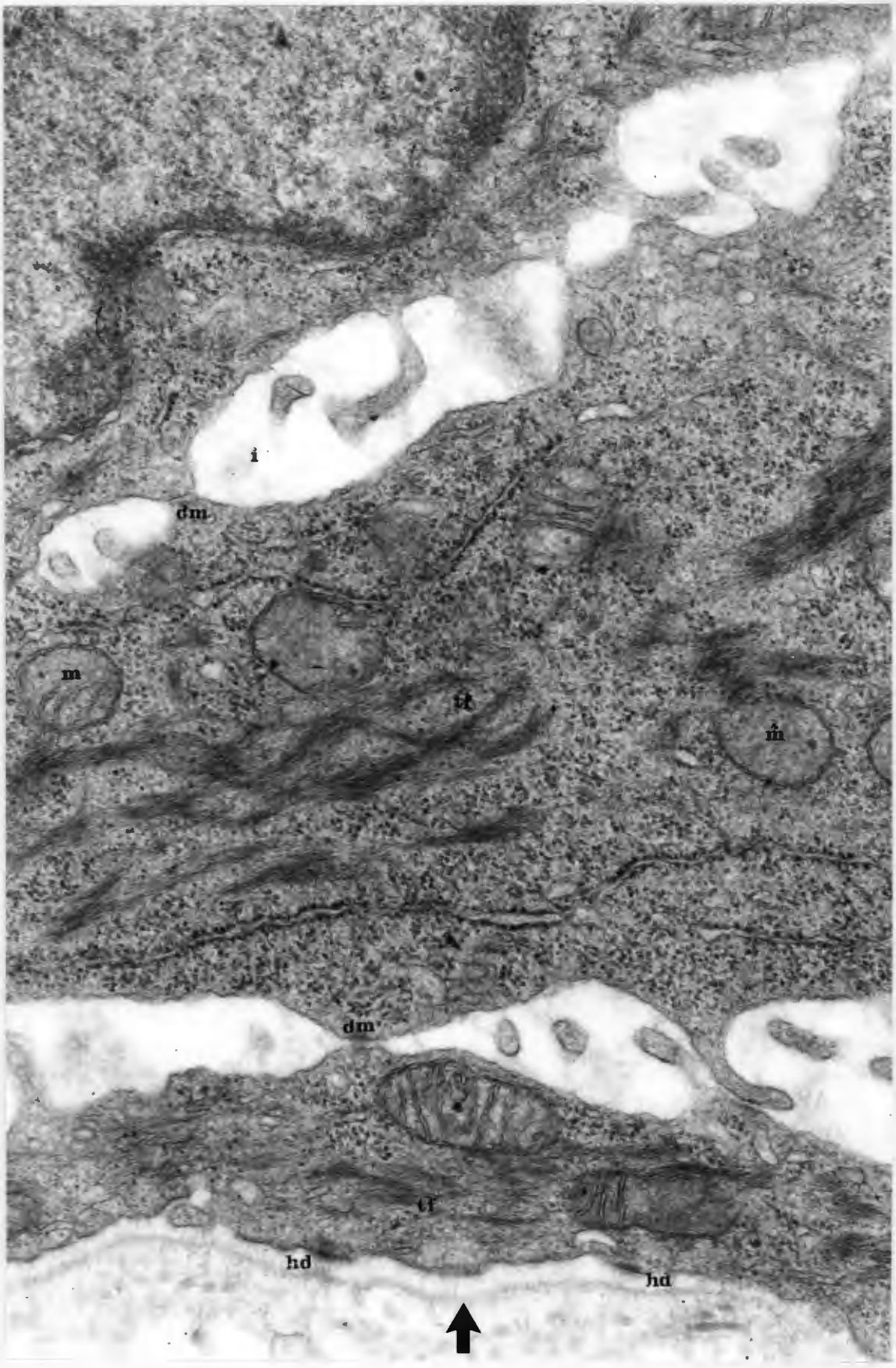
i
dm
m
tt
m
dm
tf
hd
hd

Figure 74. Lymphocytes in a perivascular situation below a hyperplastic epithelium. (H&E X 200).

Figure 75. Lymphocytes infiltrating into a nodular tumour. (H&E X 200).

Figure 76. Mast cells lying in the connective tissue of a large, mainly solid tumour. (Giemsa X 200).

Figure 77. Electron microscopic illustration of a mast cell lying within a tumour, showing dense granules. Several tumour cells are present one of which shows telolysosomes (tl). (X 12,000).

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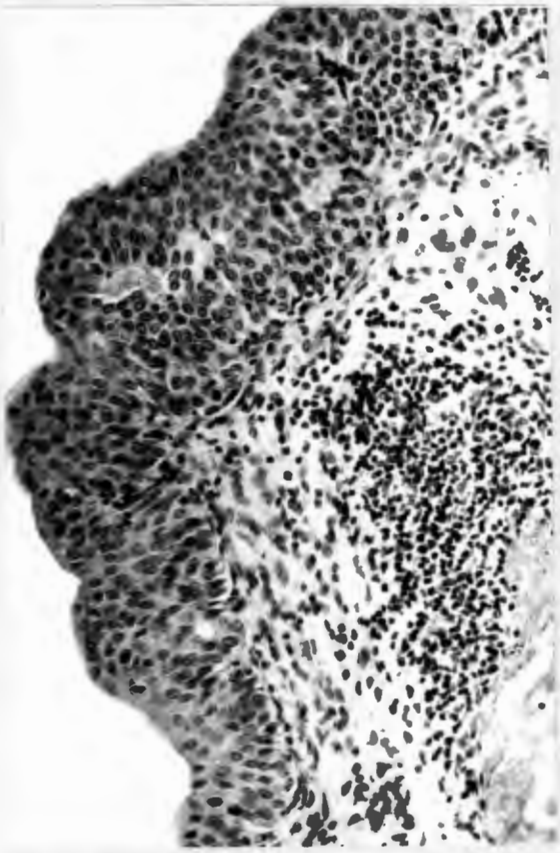

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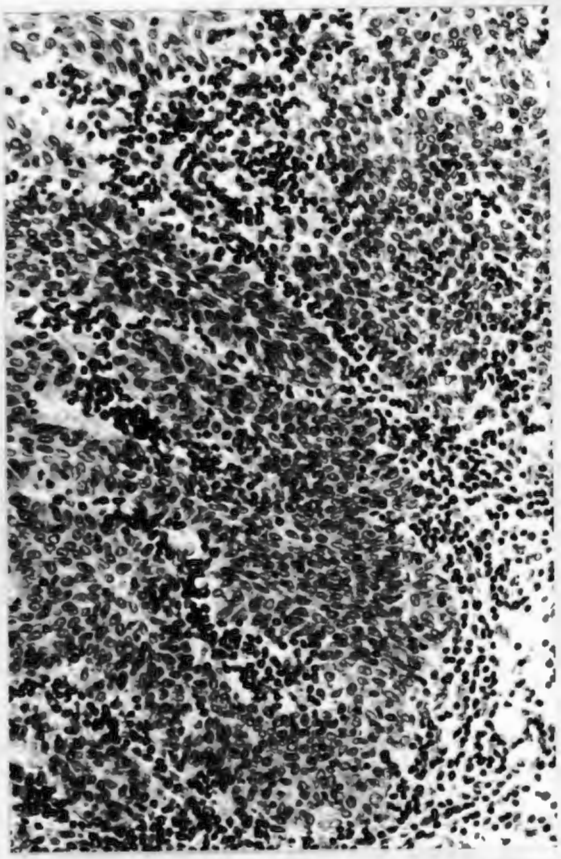

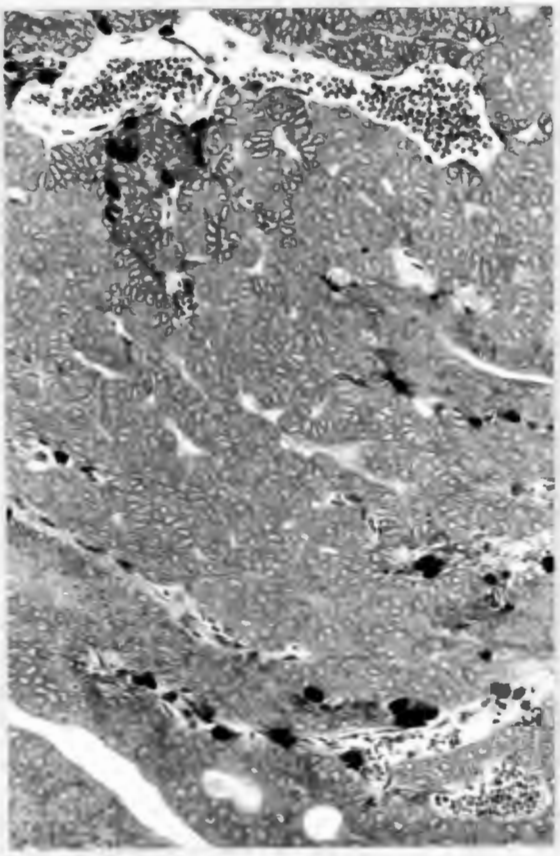

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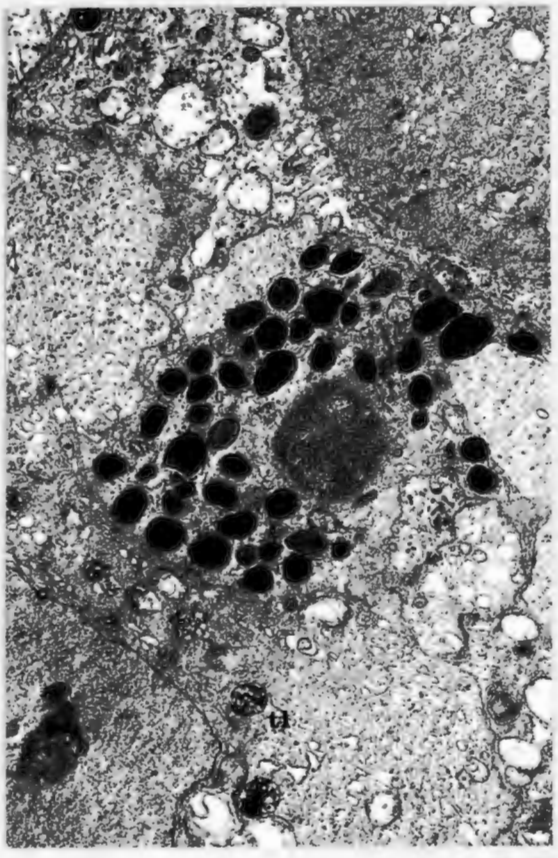

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