ULTRASTRUCTURAL INVESTIGATIONS OF NORMAL UROTHELIA AND TRANSITIONAL CELL CARCINOMAS OF RAT AND HUMAN BLADDER

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Declaration

I certify that this thesis is my own original work and that the work it contains, without direct references, has been composed by myself.

MOHAMED F.M. ISMAIL.
ABSTRACT

Normal bladder urothelia have been studied in three age groups of rats and of humans. There are species differences, the most important of which is the extent of manifestation of the specialised, ultrastructurally asymmetric membrane in the form of typical fusiform, cytoplasmic vesicles and/or as semi-rigid plaques at the luminal surface. In humans, these are never as prominent as in rats.

Age-related changes in humans are much more pronounced than in rats. One of the main changes in man is the decreasing thickness of the urothelium. The reduction in the number of cell layers in the urothelium of the elderly suggests a probable decline in the proliferation of stem cells. With increasing age, there is also a decline in the production of the specialised membrane. In the elderly, the production of this membrane has practically ceased and the luminal surface is limited by a flexible membrane with a symmetrical structure. The surface also displays simple, sparsely distributed microvilli.

The induction of bladder tumour in rats by N-methyl-N-nitrosourea has been examined in order to evaluate criteria of neoplastic transformation. Attention has been drawn to the possibility of erroneously interpreting surface features observed in exclusive scanning electron microscope studies of aged urothelia as indicators of impending neoplasia. Non-papillary tumours in man show striking ultrastructural alterations but these tumours have no counterparts in the experimental model. Ultrastructural criteria of neoplasia derived from the animal model involving the number and nature of microvilli as well as the quality and quantity of glycocalyces are applicable to low grade papillary carcinomas of human bladder. A careful analysis of these features should also allow distinction between the grades I and II.
Lectin-mediated adsorption of red blood cells and haemocyanin molecules at the luminal surface of normal human urothelia provide a further indication of changes associated with age. Binding of certain lectins to the surface of low grade papillary carcinomas is considerably higher than in normal urothelia. This could provide the basis for developing a rapid histopathological method for early and unambiguous detection of the low grade carcinomas.
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SUMMARY

The ultrastructure of normal urinary bladder urothelium has been investigated in 3 age groups of Wistar rats. The 3-layered condition of urothelium established in 10 day old rat is maintained throughout the life span.

Morphometric analyses show that the areas of the 3 cell types and their nucleo-cytoplasmic ratios are practically unchanged in the 3 age groups studied. There are, however, differences in the volume densities of some organelles in the superficial and intermediate cells of different age groups.

The most important changes in relation to age occur in the superficial cells, their Golgi apparatus and the luminal surface. The production of asymmetric unit membrane appears to be maximal in the 10 month old rat. The characteristic, scalloped contour of the luminal surface is most widespread at this age in the life of the animal.

The nature of the membrane emerging from the Golgi complex seems to change gradually with further ageing, so much so, non-angular regions with a smooth and wavy contour and symmetric membrane structure begin to make their appearance at the luminal surface in 20 month old animals. Animals nearer the end of their natural lives, however, remain to be examined.

The ultrastructural investigation of normal bladder urothelium in human has shown that age-related changes are more pronounced than in the rats maintained in the laboratory.

The thickness of the urothelium with 5-6 layers of cells is maximal in the young adult, and it decreases with age. In the aged urothelium, it may be only 1-2 cells thick in places. It would seem
that during ageing there is a decline in the proliferative capacity of stem cells and/or a decline of the stem cell population.

Morphometric analyses show that the sizes of the superficial, intermediate and basal cells and the nucleo-cytoplasmic ratios of these cells are not significantly different in the urothelia of the young adult and the middle-aged. The unusually large and highly differentiated superficial cells as present in the adult and middle-aged urothelia are not met with in the aged sample.

The most notable difference between the human bladder urothelium and that of the rat is in the luminal surface. The specialised, asymmetric membrane plaques are never so prominent in humans as in rats. This may be a species difference which had not so far been recognised. Within the proviso of the limited amount of the specialised membrane in humans, its frequency is highest in the young adult and probably also in children. During ageing there is a progressive decline in the frequency of the specialised membrane, and the luminal surface appears to become more and more flexible with a symmetric plasma membrane. In the elderly, the production of asymmetric membrane has almost ceased and the luminal surface displays sparsely distributed microvilli.

MNU-treated rat bladder guaranteed to produce papillomas and tumours have been examined in order to evaluate criteria of neoplastic transformation that may be applicable to human urothelia.

Toxic changes and large scale desquamation within 1 week of the treatment were followed by recovery and regeneration leading to a hyperplastic urothelium at 4 weeks post treatment. But, at this stage, there are no incontrovertible ultrastructural features to distinguish this preneoplastic hyperplasia from benign hyperplasia.

At 12 weeks, papillomas are formed in which the superficial layer of cells continue to exhibit signs of differentiation. The most
significant indicators of neoplastic transformation are the many microvilli and the simultaneous materialisation of a glycocalyx that could be visualised in routine preparations. Another highly significant change is in the interface between the urothelium and the lamina propria involving proliferating blood capillaries.

The papillary tumours observed at 24 weeks post MNU are characterised by extremely pleomorphic microvilli at the luminal surface which are also coated with a much thicker glycocalyx.

Spontaneously occurring transitional cell tumours in humans have been studied. These included non-papillary intraepithelial tumours for which no counterparts exist in the animal model. Various ultrastructural features of the lesions have been described, including significant alterations in the sub-mucosa which suggest their potentially invasive nature.

The ultrastructure of grade I and II papillary carcinomas have been described and compared with the normal urothelium in subjects of the age group at which the carcinomas are common. Criteria are available to distinguish the microvillous luminal surface of aged urothelium from the microvillous surface of the low grade carcinomas. The possibility of erroneously interpreting aged human urothelium as preneoplastic, on the basis of scanning electron microscope criterion relating to the luminal surface derived from the animal model, has been discussed.

In grades I and II carcinomas, some feeble signs of differentiation are evident in the superficial cells of the hyperplastic urothelium. The luminal surfaces in these carcinomas show progressively increasing numbers of microvilli and amounts of glycocalyx visualised in routine preparations as in the animal model. Examination of a reasonable number of tissue blocks of biopsies should enable an observer to distinguish between the low grades. Large scale pleomorphism of microvilli
and a much thicker glycocalyx are usual in grade II carcinoma. A continuum of other ultrastructural changes occur both within and between tumour grades. In some biopsies of low grade carcinoma, evidence exists for the presence of sub-classes of cells which may have prognostic significance.

The region of the lamina propria awaits further study in deeper biopsies before a comparison can be made with the sub-mucosa of the animal model of carcinogenesis. Various alterations observed at the urothelial-mesenchymal junction in both experimental rat tumours and spontaneous human tumours have been discussed in terms of their significance as markers of neoplastic transformation.

Lectin-mediated adsorption assays of red blood cells and haemocyanin molecules have shown some binding of 3 of the 4 lectins used as probes in aged urothelium and practically no binding in young urothelium.

Binding of lectins with different saccharide specificities is vastly greater in low grade papillary tumours compared with normal urothelia. On this basis, it may be possible to develop a rapid histological method to detect early papillary carcinoma in human bladder.
A Comparative Account of the Urinary Bladder

Ammonia, which is the main nitrogenous waste in many aquatic animals, must be eliminated from the body while at low concentration, or it becomes a cellular poison. Because of this, large volumes of water are lost whenever ammonia is the excretory product. While this presents no problem for the aquatic vertebrates, it would spell death from dehydration for a terrestrial environment. Terrestrial animals had to develop an excretory system which would enable them to eliminate metabolic waste products, while at the same time conserving water. Two different pathways for elimination of nitrogen thus developed: Uricotelism, in which the end product is uric acid and Ureotelism, in which urea is the excretory product. The factors influencing the development of a ureotelic, rather than a uricotelic metabolism have been discussed in detail by Needham (1931 and 1942) and Baldwin (1937).

The metabolism of most reptiles and birds, evolved to produce uric acid - an insoluble compound - as their main nitrogenous waste. In these animals, the uric acid leaves the kidneys in liquid urine which is discharged directly into the rectum which is fused with the ureters to form a single organ, the cloaca. The cloaca, like the mammalian rectum, is able to reabsorb water actively, and as a result a semi-solid paste of mixed excreta and urine is formed, in which the uric acid crystallizes and is then excreted, carrying with it an absolute minimum of water. The importance of this mechanism of water retention has been demonstrated by experiments on the domestic
fowl in which ureters were exteriorized, so allowing the urine to be discharged directly without entering the cloaca. The birds rapidly lost weight and died, with evidence of extreme depletion of body water despite increasing their water intake (Hart and Essex, 1942).

Mammals, on the other hand, excrete their nitrogenous waste product as urea, which is freely soluble but relatively non-toxic, except at very high concentration. Urea can hence remain in the body for some time before it is excreted.

Amphibia, such as the frog and toad and chelonian reptiles such as the turtle, also developed a ureotelic metabolism and are interesting because, like mammals, they possess a urinary bladder. However, the embryological development, function and structure of the amphibian and chelonian bladder show it to be a totally different organ from that found in mammals.

The amphibian bladder: It is an expansion of the cloaca which is formed by fusion of the ureters and the rectum. The origin of the bladder epithelium - whether it is derived from the ectoderm or the endoderm of the embryo - still remains uncertain, but it is known to have the same distribution of electrical potentials as does the skin of amphibians, which clearly is an ectodermal derivative (Gilles-Baillien and Schoffeniels, 1971).

This, thin walled sac lying free in the peritoneal cavity is capable of accommodating large volumes of urine - up to 30 ml in the toad, *Bufo marinus* (Choi, 1963). For the most part, the bladder lumen is lined by a single layer of epithelial cells supported on a basal lamina (Keller, 1963). Occasionally, a layer of basal cells has been noticed forming an interrupted second layer (Di Bona *et al.*, 1963).
Four types of epithelial cells have been distinguished: granular, mitochondria-rich, goblet, and basal cells. The granular type is the predominant type. The ultrastructural features of these cell types have been well described (Choi, 1963; Ferguson and Heap, 1970; Gfeller and Walser, 1971). The submucosa which supports the epithelium contains blood vessels and bundles of smooth muscle, and the peritoneal surface is covered by a serosa.

The amphibian bladder epithelium reabsorbs sodium salts from the urine (Leaf et al., 1958), and, it also serves as a reservoir from which the animal may absorb water during periods of water shortage (Bentley, 1966; Leaf, 1967). These features are in striking contrast to the function of mammalian urinary bladder, which normally stores hypertonic urine and from which, it is generally believed, there is almost no reabsorption of water. The permeability of the amphibian bladder has been shown to be under hormonal control and there is an extensive literature relating permeability, hormonal control, and the electrical characteristics of this organ (Dicker, 1970; Handler et al., 1972).

The Chelonian bladder: It is also a diverticulum of the cloaca but with a thicker and more muscular wall than that in the toad. The epithelium lining the bladder of this reptile is endodermal in origin, and its electrical behaviour is the same as that of its endoderm-derived intestinal epithelium, but different from that of the probably ectoderm-derived bladder epithelium of the amphibians (Gilles-Baillien and Schoffeniels, 1971).

Functionally, the chelonian bladder resembles the amphibian bladder since it reabsorbs both water and sodium salts (Bentley, 1962; Klahr
and Bricker, 1964; Nakagawa et al., 1967; and Le Fevre et al., 1970, 1971; Gilles-Baillien and Schoffeniels, 1971). The structure of the epithelium consisting mainly of columnar cells and a few other types of cells including ciliated cells, has been described by Rosen (1970) and Le Fevre et al. (1970, 1971).

The mammalian bladder: This is a unique organ without a real counterpart in other vertebrates and it evolved together with the rest of the mammalian urinary system. The primary function of the bladder is to retain urine, which is produced continuously by the kidneys, until it can be voided voluntarily at an appropriate time. The urine arriving at the bladder is frequently hypertonic and differs markedly in its organic and inorganic content from blood. In the normal man, for example, the tonicity of urine may be 2 to 4 times that of blood plasma. Unlike blood it contains no protein or glucose, but may have up to 100 times the concentration of urea and creatinine, 60 times more sulphate ions and 30 times more phosphate ions (Hicks, 1975).

Lying directly below the urinary epithelium (urothelium) lining the bladder lumen is the lamina propria containing the blood capillaries. The isotonic blood plasma is therefore only a few microns away from the hypertonic urine in the bladder lumen. The urothelium is believed to function as a permeability barrier to water and solutes, and in normal mammalian urinary bladder, the osmotic gradient that is established by the kidneys between urine and plasma remains essentially unchanged as the urine is stored in the bladder before excretion. This barrier function of the mammalian bladder is in contrast to the function of the amphibian, and chelonian bladder from which water may be absorbed.
Hicks (1975) has pointed out that this fundamental difference in function is reflected by differences in structure which set apart the mammalian bladder from that in other orders.

Clayson (1975) has pointed out that the classical idea that mammalian bladder is a relatively impermeable container for unexcreted urine has not stood the test of time. The urothelium does not possess the active transport mechanisms found in the amphibian bladder, and diffusion is a passive process. As a result of recent studies involving radioisotopes, the movement of physiological substances from the lumen of the bladder into the body and from the body tissues into the lumen of the bladder have been studied in mammals such as dogs, rabbits, rats, and man. Cooper (1975) has summarized these results and concluded that the permeability of mammalian bladder is not as low as may have previously been assumed. It is said to be about a hundred times more permeable than skin and has the same degree of permeability as several other epithelia in the body which do not exhibit active transport. Experiments with animals indicate that bladder permeability is profoundly altered if the integrity of the specialised luminal plasma membrane or the tight junctions between contiguous superficial cells is destroyed (Hicks, 1966a; Turnbull, 1971). The luminal membrane and junctions have also been shown to be defective during carcinogenesis and tumour-containing bladders (Clayson, 1975; Merk et al., 1977).

The embryology of mammalian bladder: This aspect has been reviewed in detail by Hutch (1972). Developmentally, the mammalian bladder is a complex structure: its main part originates in the early embryo from the cloacal endoderm but the trigone is formed from the mesodermal epithelium of the Wolffian duct. The urinary face of the trigone is
covered with the same type of epithelium as the rest of the bladder.

The structure of the mammalian bladder: The gross anatomy of the bladder has been reviewed recently (Hutch, 1972; Hicks, 1975). The bladder wall has four parts: serous, muscular, lamina propria or submucosa and the urothelium or the mucosa. Outermost is the serosa which contains the mesothelial lining of the peritoneum and a loose connective tissue. Next, there is the muscular coat which consists of three layers of smooth muscle. The outer and inner layers are arranged longitudinally while the middle layer is arranged in a circular or spiral manner. The third part is the lamina propria formed of loose, fibrous, connective tissue in which blood vessels, nerve fibres and occasional reticulo-endothelial cells are located. The blood capillaries are close to the base of the urothelium and often indent the basal lamina. The epithelium rests on the thin, uninter rupted basal lamina which cannot be resolved by the light microscope. It is, however, readily seen in the transmission electron microscope. The epithelium is a modification of the stratified, cuboidal type referred to as "transitional" epithelium and more recently (Melicow, 1945) as urothelium. The urothelium is thin relative to the other parts of the wall of the bladder, and it may have a variable number of cell layers in different species.

The bladder urothelia of the rat and mouse are very similar and the ultrastructure of these urothelia has been described by various workers (Walker, 1960; Leeson, 1962; Richter and Moize, 1963; Hicks, 1965, 1966a,b, 1975; Koss, 1967, 1969; Firth and Hicks, 1973; Clayson, 1975; Hicks et al., 1976; Newman and Hicks, 1977; Pauli et al., 1977; Hicks and Chowaniec, 1978; Phillips and Davies, 1980). From these
studies, a general picture of the organization of urothelium has emerged. It consists of three to four layers: a basal layer of small cells, one or two intermediate layers of medium-sized cells, and a peripheral or superficial layer of very large cells.

The basal cells have a relatively simple cytoarchitecture. They contain a moderate number of mitochondria and the Golgi apparatus is poorly developed. They have little endoplasmic reticulum but contain many free ribosomes forming the bulk of the protein-synthesizing system in the cytoplasm. The nuclei of these cells are diploid and mitotic figures are very occasionally seen. They are attached by half desmosomes to the underlying basal lamina at frequent intervals, and by desmosomes to the adjacent basal and intermediate cells.

The intermediate cells, as their name implies, are intermediate both in position and in degree of differentiation between basal and superficial cells. They are relatively large and are thought to be tetraploid (cf. Clayson, 1975). Their cytoplasm is usually less basophilic than the basal cells. The assembly of thick membrane (see later) commences in these cells and their Golgi complex shows some degree of specialisation. Some fusiform or discoidal vesicles as well as lysosomes are found in their cytoplasm, but their plasma membrane is composed of uniformly symmetrical unit membrane. It is generally accepted that the undifferentiated basal cells and the partially differentiated intermediate cells are sources of replacement for the superficial layer of cells.

The superficial cells are considerably larger than the intermediate cells and are highly differentiated. The surface cells are generally believed to be highly polyploid - octaploid or higher - especially in mice and rabbits, and Levi et al. (1969) have reported that binucleate cells are often present. The free or luminal surface
of the superficial cells of the urothelium is limited by a plasma membrane with remarkable ultrastructure. This membrane is considered to be a unique feature of the mammalian (as exemplified by rat) urinary bladder (cf. Hicks, 1975). It has been estimated (Porter et al., 1967; Staehelin et al., 1972) that about 73% to 90% of its area is thicker than the rest. The thickened regions measure about 12 nm across and appear to form semi-rigid plaques, separated by 8 nm thick membrane. The plaques are uniquely asymmetric in that the outer lamina, that is the lamina of the membrane facing the bladder lumen, or the outer leaflet of the unit membrane structure is approximately twice as thick as that of the inner lamina facing the cell cytoplasm (Hicks, 1965; 1966a,b; Koss, 1969). The semi-rigid plaques made up of asymmetric unit membrane are assumed to be joined by flexible "hinges" of symmetrical inter-plaque membranes. Isolation of the plaque membrane, and electron microscope examination of negatively stained fragments have confirmed earlier indications of subunits in the form of twelve particles arranged in a stellate pattern with a centre-to-centre spacing between them of 12 to 13 nm (Hicks and Ketterer, 1969; Vergara et al., 1969; Staehelin et al., 1972; Hicks et al., 1974). Further aspects of the unusual structure of this asymmetrical membrane and the hexagonal lattice arrangement of its subunits have been described by Hicks (1975).

Chemical analyses show that the protein-lipid ratio of the luminal plasma membrane is low compared with that of other membranes, with the exception of myelin, and approximately two-thirds of the volume of the luminal membrane is composed of lipid and only one-third of protein (Vergara et al., 1974). Chromatographic analysis of its lipids shows 4 major components to be present, namely cholesterol, phosphatidyl choline, phosphatidyl ethanolamine and cerebroside. The latter is a
very unusual component to be found in quantity in an epithelial cell membrane (Ketterer et al., 1973; Hicks et al., 1974; Vergara et al., 1974; Hicks, 1975). Although only one third of the volume of the asymmetric membrane is protein, this component may play an important role in organising the structure of this membrane. Amino acid analysis of the protein shows (Ketterer et al., 1973) a high proline content. Relatively little is known as yet of the carbohydrates of this membrane, but this component is likely to prove to be of interest, especially the galactoside head group of the polar lipid, cerebroside (Hicks, 1975).

The apical cytoplasm of the superficial cells of rats contains numerous empty-looking fusiform or discoidal vesicles of various size, that are lined by asymmetrical membrane. These vesicles are particularly abundant along the luminal membrane and in the vicinity of well-developed Golgi complexes. Some vesicles are continuous with the luminal membrane and are hence open to the lumen of the bladder; these possibly provide extra surface capacity to allow for changes in bladder volume during filling and emptying. The process has been described by Hicks (1975). It is believed that as the bladder contracts on micturition, the so called hinge regions of the luminal membrane enable the membrane to fold and invaginate into the cytoplasm to form the large fusiform vesicles bound by asymmetric membrane. The luminal surface area is thereby rapidly reduced when the bladder contracts and the vesicles serve to store the membrane in the cytoplasm. During dilation of the bladder, the reverse process occurs; the vesicle membrane is reinserted into the surface by fusion of the thin hinge regions with like areas in the luminal membrane. Koss (1969), however, has disputed the view, that fusiform vesicles are even formed by invagination of the luminal membrane during contraction of the bladder. He has argued that the asymmetric
membrane forms only large canals and communicating vesicles and that these provide the reserve membrane for use during dilation of the bladder. Both these workers (Hicks, 1966b; and Koss, 1969) agree that the asymmetric luminal membrane is assembled in the Golgi complex and transported to the cell surface in the form of vesicles.

Immediately below the luminal membrane, the cytoplasm contains a network of cytoplasmic filaments, the "terminal web" of earlier histological descriptions, which resemble the α-keratin fibrils found in keratinizing tissues such as skin and buccal mucosa. The cytoplasm of these cells also contains rough endoplasmic reticulum, free ribosomes in moderate quantity, abundant mitochondria and numerous large, densely staining bodies which can be identified by their enzymic content as derivatives of lysosomes, which have been referred to in the cytological literature as telolysosomes, autophagic vacuoles, heterogeneous bodies, or polymorphic bodies. They have been seen to contain remnants of asymmetrical membranes. The plasma membrane of the superficial cells interdigitate with their neighbours and with cells in the intermediate layer in a complex manner, but there are few points of intimate contact. Maculae adherentes - junctions which are widely known as demosomes - are observed at various intervals along the borders. The superficial cells are further stabilized by zonulae occludentes or tight junctions between the lateral membranes near their luminal border. These tight junctions act as a diffusion barrier between the urine and the tissue fluids of the intercellular cisternal space of the urothelium (Hicks, 1966a).

Scanning electron microscopy, although limited by its inability to reveal more than the surface of the tissue, provides an extraordinary view of surface topography and the great depth of focus it
offers, makes it an important tool in the study of the cell surface. Relatively large tissue specimens, as compared to the size of the specimens examined by transmission electron microscope, can be examined with a magnification ranging from X 30 to almost X 100,000. The data gathered by this means can be correlated with the findings of transmission electron microscope examination to yield significant information about the surface of the tissue. The surface characteristics of normal rat and mice urothelia have been investigated by several workers (Hodgson, 1968; Burke, 1976; Hodges et al., 1976, 1977; Jacobs et al., 1976; Newman and Hicks, 1977; Hodges, 1978; Phillips and Davies, 1980). The surface layer is seen to consist of fairly regular, large, six-sided epithelial cells averaging 25 \( \mu \text{m} \) by 15 \( \mu \text{m} \) with tight cell-to-cell contact margins visible as ridges with a central groove. Raised angular foldings of the cell membrane characterize these cells, giving an irregular and variably ridged structure to the luminal surface depending on the extent of bladder distension. One or two small globular processes may extend within the convoluted membrane infoldings. Desquamation of one superficial cell exposes several smaller cells, of the intermediate layer, averaging 6 \( \mu \text{m} \) by 5 \( \mu \text{m} \). The immature intermediate cell has irregularly distributed, stubby globular projections and larger blebs.

**Turnover of bladder urothelium.** A remarkable feature commented upon by several investigators is the stability of bladder urothelium observed in laboratory rodents. The rate of turnover of normal, undamaged urothelium is very slow, and mitotic figures are seldom seen in the adult bladder. It has been reported (Clayson and Pringle, 1966; Levi et al., 1969; Cooper, 1972; Martin, 1972) that only one cell in ten thousand
is in division at any given time. Autoradiographic studies following supply of tritiated uridine indicate that cells of the bladder urothelium in guinea-pigs and rats may have a life span of about two hundred days (Levi et al., 1969; Cooper, 1972; Martin, 1972).
1. Materials

Normal Human Urothelium and Tumours. The normal samples were obtained with Storz forceps. Biopsies were obtained from young adults and middle-aged and elderly subjects who were being investigated for conditions such as nocturnal enuresis, urothelial abnormalities urgency and bladder incontinence.

The papillary tumour biopsies were also obtained with Storz forceps, mainly from elderly subjects who had no other symptoms suggestive of infection or urinary obstruction, and the samples were taken before any form of treatment was initiated. Samples of atypical areas adjacent to grossly visible tumours were taken from some of the patients. The tumour biopsies and the samples suspected to be carcinoma in situ were initially checked by light microscopy. The light microscope examination and tumour grading were carried out by the Pathology Department of the Western General Hospital. I am indebted to Drs. J.N. Webb and A. Busuttil and their staff for their help and co-operation.

The bladders were washed and moderately distended with normal saline before the biopsies were taken. I am indebted to Messrs. W.S. Tulloch, G. Carswell, T.B. Hargreave, J.E. Newsam, C. Ludgate, J.R. Hindmarsh, D.N.A. Wallace, G.D. Chisholm and G. Smith and their theatre staff for the collection of biopsies. The tissue samples were immediately fixed in the operating theatre and taken to the laboratory for further processing.

Normal Rat Urothelium. The Wistar rats for this study were supplied by the Animal House of the Wilkie Research Laboratory, Department of
Surgery, University Medical School. The animals were free of endo- and ecto-parasites, protozoa, *Salmonella*, and other important pathological bacteria. They were fed a commercial cereal-based diet, supplemented with vitamins and minerals with a metabolizable energy content of 10258 J/g, 17.7% crude protein, 4.2% crude fat and 6.8% crude fibre. Tap water was always available. The 10 day old rats were nursed at the breast for ten days before sacrifice.

The bladders were moderately distended and fixed *in situ* with glutaraldehyde before they were removed and bisected for further processing.

**Rats for Induction of Bladder Tumours.** These experiments were performed at the Animal House in the Western General Hospital. The Wistar rats used were 9 months old at the start of the experiment. The experimental procedures, except anaesthetising and catheterising the rats, were carried out in the fume cupboard. A large beaker of alkali was provided in the cupboard. The rats were anaesthetised using Hypnorn (0.2 mg/ml Sentanyl and 10 mg/ml Fluanisone) and Diazepam and were catheterised with a 2FG Portex catheter. Each dose (1.5 mg MNU in 1 ml solution) was instilled into the bladder for a period of 30 minutes. The bladder was then washed out with normal saline and the animals allowed to recover. The bladder washings were put into alkali. The rats spent the next 24 hours in the fume cupboard and were then transferred to clean cages. The bedding material was destroyed and the cage washed well with alkali. The procedure was repeated three more times biweekly. After completion of the fractionated course of treatment, the animals were sacrificed at 1 week, 4 weeks, 12 weeks and 24 weeks intervals and the bladders
fixed in situ as mentioned before.

2. Methods

Transmission Electron Microscopy. Tissues were fixed in a cold (1 - 4°C) 5.0% solution of glutaraldehyde (EM grade, specially purified for use in electron microscopy, supplied by TAAB Laboratories, Reading) in 0.1 M cacodylate buffer, pH 7.2, for about 1 - 3 hours. While still in the fixative, the tissues were cut into small, approximately 1 mm³ pieces, and rinsed overnight in the buffer in the fridge. This was followed by post osmification in cold, 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 3 hours when it was allowed to warm up to room temperature. The samples were then briefly washed in the buffer, dehydrated through a graded series of ethanols and taken through propylene oxide into a freshly prepared mixture of Epon and Araldite. After infiltration overnight in the Epon-Araldite mixture at room temperature on a shaker, the tissues were placed in suitable moulds containing the epoxy resin mixture and oriented under a dissecting microscope. Polymerisation was carried out in an oven at 58-60°C for about 40 hours. The Epon-Araldite mixture used was a modification of an embedding medium proposed by Mollenhauer (1964). The mixture consisted of the following proportions.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epon 812</td>
<td>15.00 ml.</td>
</tr>
<tr>
<td>Araldite CY212</td>
<td>12.00 ml</td>
</tr>
<tr>
<td>Araldite Hardener HY 964</td>
<td>36.00 ml.</td>
</tr>
<tr>
<td>Araldite Accelerator DYO64</td>
<td>1.26 ml.</td>
</tr>
</tbody>
</table>

After trimming the blocks, 1 μm thick sections were cut with a Porter-Blum MT-1 ultramicrotome using glass knives. The sections were mounted on glass slides, stained with 3% basic Fuchsin in 70% alcohol
and the orientation of tissue was checked by light microscopic examination. Only when the block was suitably oriented, was it taken up for ultrathin sectioning: Ultrathin sections showing silver-pale gold interference colours were cut, mounted on copper grids which had been previously covered with a carbon-coated Formvar membrane. The sections were stained for 60 minutes in 1% uranyl acetate in 25% ethyl alcohol. The stain was freshly prepared just before use from a stock solution of 2% uranyl acetate in distilled water. After staining with uranium, the grids were rinsed 3 times in 30% ethyl alcohol and taken up for further staining in lead citrate as described by Reynolds (1963). Drops of lead citrate were placed on a layer of wax in a Petri dish and the grids with sections facing down were floated for 15 minutes with the usual precautions to prevent the formation of lead carbonate. After staining, the grids were washed first in 0.01 N NaOH and then in distilled water and dried. The double stained sections were examined in an AEI-EM6 electron microscope operating at 60 kV.

Staining with Ruthenium Red. The tissues were fixed for 1 hour at room temperature in the following solution.

\[
\begin{align*}
3.6\% \text{ glutaraldehyde in water} & \quad 0.5 \text{ ml.} \\
0.2\% \text{ Cacodylate buffer, pH 7.3} & \quad 0.5 \text{ ml.} \\
\text{Ruthenium red stock solution} & \quad 0.5 \text{ ml.} \\
\text{(from TAAB Laboratories)} & \\
\end{align*}
\]

The stock solution was made up according to the formula of Luft (1971). About 30-35 mg. of Ruthenium red was weighed out and crushed in a small mortar with a few drops of distilled water. More water was added and the solution transferred with a Pasteur pipette to a graduated 15 ml. centrifuge tube with successive washing of the mortar until
sufficient water was added to make up a final concentration of 10 mg/ml. This suspension was then heated in a water bath at 60°C, for 5 minutes with frequent agitation and then centrifuged at 1600G for 5-10 minutes. The supernatant was carefully removed and stored in a refrigerator as the stock solution.

After fixation, the tissues were rinsed with 3 changes of 0.15 M cacodylate buffer over a period of 10 minutes and post-fixed for 3 hours in the following solution, also at room temperature.

\[
\begin{align*}
2\% \text{ Osmium tetroxide in water} & \quad 0.5 \text{ ml} \\
0.2 \text{ M Cacodylate buffer, pH 7.3} & \quad 0.5 \text{ ml} \\
\text{Ruthenium red stock solution} & \quad 0.5 \text{ ml}.
\end{align*}
\]

The tissues were then rinsed briefly with buffer, dehydrated as usual and embedded in epoxy resin mixture. Brief staining of thin section with Reynold's lead citrate was found useful.

**Morphometry.** Transmission electron microscopic morphometry of the urothelial cells was carried out according to the methods described by Weibel (1969). A random sample of three blocks from each of the samples to be analysed was used to determine the cell and nuclear areas and the volume density of the cytoplasmic organelles. For the cell and nuclear areas, pictures were taken at an initial magnification of X 3,000 and enlarged to X 6,000. A 9:1 double lattice was superimposed on each micrograph and the number of squares overlying the cells and the nuclei was counted using the coarse mesh (2.25 cm²). The problem of rounding off was made easier by marking the centre of each square; when the centre point was inside the profile the square was counted as one, but when it was outside it was rejected.
The cell and nuclear areas were estimated as follows:

The profile area in \( \mu m^2 \)

\[
= \frac{\text{The number of squares inside the profile} \times 225 \times 10^6}{6000 \times 6000}
\]

where \( 225 \times 10^6 \) is the area of the coarse mesh in \( \mu m^2 \) and 6000 is the final magnification of the photograph.

A second sample of electron micrographs of the same sections used for the cell and nuclear areas was recorded at initial magnification of X 10,000 and enlarged to 30,000. The volume density of Golgi bodies and vacuoles, mitochondria, endoplasmic reticulum, polymorphic bodies and cytoplasmic matrix in the superficial, intermediate and basal cells was estimated by superimposing on each micrograph a lattice of 100 points. The number of points overlying different organelles was counted in order to determine their relative volumes and each organelle was expressed as a fraction of the total number of points in each micrograph.

**Bladder carcinogen.** \( N \)-Methyl-\( N \)-nitrosourea (MNU) was obtained from Sigma (London) Chemical Co. Ltd. It was kept at the Animal House at the Western General Hospital, and stored under nitrogen at -20\(^o\)C in a clearly labelled container. The MNU stock solution was made up into solution containing 6 mg/ml. The solution was prepared only when required and was made up in the fume cupboard by Dr. N.F. Anderson. My thanks are also due to Dr. N.F. Anderson for carrying out the actual instillations into the rat bladder.

**Scanning Electron Microscopy.** Tissues were distended gently and fixed with 2.5% solution of glutaraldehyde in 0.1 M cacodylate buffer,
pH 7.2 for 5-10 minutes at room temperature. Fixation was then continued for a further 30-36 hours before the tissues were cut into small pieces approximately 5 x 5 x 2mm³. After several rinses in the buffer, the tissues were washed in distilled water and dehydrated in a graded series of acetone in the usual manner. After dehydration, critical point drying followed according to standard procedures (Hayat, 1978). The specimens were then glued on to aluminium stubs using silver paste and with the surface to be examined facing upwards. The urothelial side of the specimen can be distinguished from the connective tissue side by its smoother and shinier surface when examined in a dissecting microscope with reflected light. When the paste had dried, the specimens were coated with a layer of gold in a sputtering diode coating unit in the prescribed manner. The samples were examined in an ISI-60 scanning electron microscope operating at 30 kV using the backscattered electron imaging. My thanks to Miss J.L. Tocher for her help in the operation of the microscope.

Lectin-mediated red blood cell adsorption to urothelial surfaces. The materials were processed according to the technique described by Furmanski et al. (1972) with some modifications. The tissues were washed three times with the PBS, flattened and slightly stretched by pins on a layer of wax contained in a Petri dish, and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 5-10 minutes. The tissues were then washed three times with PBS containing Ca⁺⁺ and Mg⁺⁺ at room temperature. The samples were incubated overnight at 4°C in 0.1 M ammonium chloride in PBS to block any remaining aldehyde groups which might otherwise cause non-specific labelling. The tissues were then allowed to stand at 37°C for two hours before being treated with appropriate volume of lectins at a concentration
of 100, 250 and 500 μg/ml in PBS at pH 7.4 and incubated for 30 minutes at 37°C. After the samples were washed three times in PBS, the erythrocyte suspension was added. After further incubation for 30 minutes at 37°C, the unadsorbed erythrocytes were removed by washing three times with PBS, and this was facilitated by tapping the side of the dish against a hard object. The test tissues were post-fixed for 30 minutes at 4°C in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, to bind the protein-marker covalently to the cell membrane, then rinsed in two changes of cacodylate buffer and distilled water and dehydrated through a graded series of acetone and processed for scanning electron microscopy as already described.

The following controls were set up. Tumour samples incubated with 500 μg/ml Con A solution containing 100 mM of α-methyl-D-glucoside and 100 mM of α-methyl-D-mannoside; tumour samples incubated with 500 μg/ml WGA solution containing 200 mM N-acetyl-D-glucosamine; tumour samples incubated with 500 μg/ml PHA solution containing 200 mM N-acetyl-D-galactosamine; and tumour samples incubated with 500 μg/ml PWM solution containing 200 mM N-acetyl-D-glucosamine.

The number of RBCs adsorbed to the surface in lectin-mediated RBCs adsorption assays were counted in 20 scanning micrographs per group at initial magnification of X 500 and enlarged to X 2,000.

The means and the standard errors for each measurement were calculated and statistical comparisons were made using the student’s t-test.

Con A - mediated haemocyanin binding to urothelial surfaces. The materials were processed according to the technique described by Smith
and Revel (1972) with some modifications. The samples were washed three times with the PBS, flattened and slightly stretched by pins on a layer of wax contained in a Petri dish and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 5-10 minutes. The tissues were then washed three times with PBS containing Ca^{++} and Mg^{++} at room temperature and incubated overnight at 4°C in 0.1 M ammonium chloride in BPS to block any remaining aldehyde groups. The materials were then allowed to stand at 37°C for two hours before treatment with the appropriate volume of Con A at a concentration of 100, 150 and 250 μg/ml in PBS at pH 7.4 and incubation for ten minutes at 37°C. After the samples were washed three times in PBS, they were labelled for ten minutes with 1 mg/ml haemocyanin in BPS at 37°C. After labelling, the samples were fixed again with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for ten minutes and 1% osmium tetroxide in 0.1 M cacodylate buffer for ten minutes. Following dehydration through a graded series of acetone, the tissues were dried from liquid CO₂ by the critical point drying method, lightly coated with carbon first and then gold and examined in the scanning electron microscope.

An appropriate control was set up as previously mentioned for lectin-mediated RBCs adsorption.

The lectins, Con A, phytohaemagglutinin, wheat germ agglutinin and Pokeweed mitogen were supplied by Flow Laboratories. α-Methyl-D-glucoside, α-Methyl-D-mannoside, N-Acetyl-D-glucosamine and N-Acetyl-D-galactosamine were supplied by Sigma Chemical Company.

Solutions of the lectins were made in phosphate-buffered saline (PBS) which was prepared as described by Molday et al. (1975): it consisted of 8.0 g NaCl, 0.2 g KCl, 0.2 g KH₂PO₄ and 1.15 g Na₂HPO₄ per litre of distilled water, adjusted to pH 7.4. The solution was then
filtered through Millex-GS filter unit (0.22 μm) supplied by Millipore S.A., 67 Molsheim, France. To remove surface serum containing saccharide from the tissue, 0.2 g CaCl₂ and 0.1 g MgCl₂ were added to the PBS (Pretton and Bariety, 1978).

Erythrocytes: Human "O" blood in Alsever's solution was obtained from Flow Laboratories. The cells were washed 3 times with PBS immediately before use and suspended at a final concentration of 2% by volume in PBS.

Haemocyanin (keyhole limpet) lyophilised, was obtained from Calbiochem-Boehringer Corporation Laboratories Ltd.
CHAPTER I

ULTRASTRUCTURE OF NORMAL BLADDER UROTHELIUM IN
RATS OF DIFFERENT AGE GROUPS

Introduction

There is a good deal of information on the ultrastructure of bladder urothelium in rats and certain other animals but there is practically no information on structural alterations that may occur with increasing age of animals. Investigations in the past have been concerned with cytodifferentiation in the three cell types and the specialised features of the luminal plasma membrane. These studies were carried out, presumably in adult animals and hardly any author has deemed it necessary to specify the age of the specimen used. Although there are reasons to suppose that age influences disease processes (Franks, 1975; Peto et al., 1975), very few investigators have attempted to examine age effects at the tissue or organ level. Exceptions are the intestinal tract (Rowlatt, 1975) and the skin (Ebbesen, 1978). The present study was undertaken to examine in a systematic manner the ultrastructure of bladder urothelium in three arbitrarily chosen age groups of rats kept in laboratory conditions. The results are to be compared with urothelia in different age groups of humans.

OBSERVATIONS

Young rats (10 days old)

Rats aged 10 days were used for this study. The slightly distended bladder of these animals showed for the most part three layers
of cells with fairly straight and closely opposed cell borders (Figs. 1, 2). In places the urothelium was seen to consist of two layers (Fig. 4). On few occasions the urothelium appeared to be four layered (Fig. 3). The basal lamina and the lamina propria were clearly seen in all cases. The lamina densa of the basal lamina was thin, measuring about 40 nm in thickness. The extracellular space was narrow especially between the superficial cells and the superficial and intermediate cells, but a little wider between the lower layers of cells (Fig. 3).

Cell junctions and contacts. The urothelial cells were interconnected by maculae adherentes (desmosomes) and the superficial cells were further stabilized near the luminal surface by zonulae occludentes (tight junctions). The tight junctions (Fig. 5) measured about 0.25 μm in length, and appeared as extended contacts where the outer leaflets of the triple layered structure of the adjoining membrane converged to form a single fused entity. The width of the fused junctional membrane was clearly less than what would be the combined widths of the two contributing unit membranes. The width of the junctional region was only 18 nm, while each of the associated membranes was about 12 nm. Immediately below the tight junctions, there was often present another region of membrane association, a zonula adherens (Fig. 5) where the two plasma membranes ran parallel to each other but were separated from each other by an interspace measuring about 15-20 nm. In addition, the cytoplasmic sides of the junction contained a fairly electron dense mat of filaments. A third kind of junction forming part of the series described above was situated further below (Fig. 5) and it possessed features characteristic of desmosomes. The junction consisted of two parallel
plasma membranes which were separated by a wide interspace measuring 35-40 nm. The interspace appeared to contain some electron dense material and the cytoplasm adjoining the junction contained dense fibrillar plaques and associated cytoplasmic filaments.

The borders between cells were smooth and closely opposed with only a few interdigitations (Fig. 3). The extracellular compartment was therefore not extensive although as mentioned earlier, it tended to be more irregular and wider towards the basal layer. Desmosomes were present more often along the lateral borders of cells than along the apical-basal borders. From examination of a large number of preparations, the impression gained was that the desmosomes between superficial cells were better developed than those between basal cells. The latter were found to be smaller with fewer microfilaments in the subjacent cytoplasm. A moderate number of hemidesmosomes were observed on the basal cell plasma membrane abutting the basal lamina (Fig. 1). On occasions, sub-basal, dense, plaque-like areas were noticed on the basal lamina facing the hemidesmosome. At the level of the basal lamina, the extracellular compartment appeared to be open, there being no junctions to isolate it from the underlying non-epithelial tissue.

The luminal surface in TEM and SEM. In low power transmission electron micrographs, the plasma membrane limiting the urinary face of the superficial cells usually showed an angular or scalloped contour, as may be seen in Fig. 1. The angular regions were separated by short slightly elevated, crest-like regions. The plasma membrane in the angular regions measured about 12 nm in thickness and showed asymmetry in its triple-layered structure. Its outer lamina facing the lumen of the bladder was thicker than the inner lamina facing
the cell cytoplasm; the asymmetry was not evident in the crest regions (Fig. 8).

In the scanning electron microscope (SEM), the luminal surface cells of dilated bladder were seen to consist of large, polygonal cells (Fig. 6). Clearly, the cells though large, are not uniform in size in the young urothelium.

At higher magnification (Fig. 7), the cell borders appeared as slightly elevated ridges, and the surface of the cells showed a very simple pattern of shallow microridges. Short grooves visible at times along the elevated border ridges are presumably the sites of junctions between adjoining cells. Another point of interest was the presence of a few small craters on the surface (Fig. 7) representing the openings of fusiform vesicles.

**Superficial cells.** These were generally the largest cells of the urothelium, although there was considerable variation in their size, as was also noted in scanning electron micrographs. The organelle content of the superficial cells was generally higher than in the intermediate cells lying below. A notable feature of the superficial cells was the presence of many fusiform vesicles especially in the peripheral cytoplasm adjoining the luminal membrane. They were limited for the most part by the same type of asymmetrical membrane as described above in the angular regions of the luminal membrane (Figs. 2, 3, 4, 8), with the thicker lamina lining the lumen of the vesicles and the thinner one facing the cytoplasm. The vesicles were most often oriented parallel to the luminal surface (Figs. 2, 3, 4, 8) and occasionally some of them appeared to be in communication with the surface (Fig. 9), as described earlier (Fig. 7) in scanning electron micrographs.
In addition to the fusiform or ellipsoidal vesicles, the superficial cells occasionally showed a few large vesicles of irregular shape as in Fig. 10, containing diffuse material; the limiting membranes of these vesicles were also asymmetrical in structure. The proximity of the vesicle to a narrow channel lined by asymmetric membranes as in Fig. 10, suggests that the vesicle had been part of the luminal surface when the bladder was stretched and that it invaginated to form a vesicle during contraction subsequently, trapping some of the surface material in the process.

Golgi apparatuses were also located in the apical or lateral portions of the cell. The few profiles of the endoplasmic reticulum that were met with, occurred in the vicinity of the Golgi apparatus. Mitochondria were present in considerable numbers in the superficial cells. Polymorphic bodies were not very common and those present were spherical to ovoid structures varying in size from about 0.5 μm to 0.8 μm in diameter. The larger ones often enclosed heterogeneous material and an electron dense matrix (Fig. 3); in high power electron micrographs, their limiting membrane was seen to be symmetrical in structure. The cytoplasmic matrix contained free ribosomes and microfilaments lying mainly parallel to the luminal surface (Fig. 3).

The chromatin in the nucleus of the superficial cells was mostly in the diffuse form and was distributed more or less uniformly. The nuclear envelope was smooth and showed no indentations. The nucleoli of these cells invariably exhibited a fibrillo-granular network structure.
Intermediate cells. In the young animals, the varying thickness of the urothelium described above, meant that there was either one intermediate layer (in 3 cell thick urothelium), two (in 4 cell thick region of the urothelium) or none (in 2 cell thick regions).

The intermediate cells in the 3 cell thick urothelium (Fig. 1) usually contained no fusiform vesicles. The Golgi apparatus was smaller than in the superficial cells. It was located in the lateral region of the cell and consisted of small cisternae and few vesicles; there was also no trace of any AUM limited profiles. A few polymorphic bodies and several profiles of endoplasmic reticulum were observed. The above description of the intermediate cells also applied to the lower of the two intermediate cells in the 4 cell thick urothelium (Fig. 3).

The intermediate cell immediately adjoining the superficial cell in the 4 cell thick urothelium resembled the overlying superficial cell in many respects (Fig. 3). The apical plasma membrane of this intermediate cell had, in general, a symmetrical structure, but there were present asymmetrical unit membrane-limited vesicles in the immediate vicinity of this membrane. Occasionally one such vesicle appeared to be in continuity with the plasma membrane (Fig. 3). The AUM-limited vesicles were, however, fewer in number than in the superficial cells. The Golgi complexes were better developed than in the intermediate cells described above and, in some sections extended profiles of rough endoplasmic reticulum were also noted. Also present were polymorphic bodies, microfilaments and free ribosomes.

The mitochondria in all the intermediate cells were usually small and rounded structures measuring about 0.25 \( \mu m \) in diameter.
But, elongated profiles measuring up to 1.2 μm were seen in some sections. In the nucleus, there was some margination of the heterochromatin.

**Basal cells.** They were the smallest of the urothelial cells and appeared generally more electron dense than the overlying cells. The cytoplasm was very rich in ribosomes and there were several mitochondria, but apart from these, the content of other organelles in the cell was low. Scattered profiles of rough endoplasmic reticulum and Golgi apparatus were discernible with the latter located invariably in the basal part of the cell. Compared with the overlying cells and especially the superficial cell, the basal cell nuclei possessed much more heterochromatin located along the margin of the nuclear envelope. The nuclear envelope was smooth and the nucleoli were prominent relative to the size of the cell.

**Morphometric analysis of the urothelial cells.** A quantitative study of the size relationships of different cell types and the organelles in them was undertaken with a view to clarifying any structure-function correlations that may exist in the tissue in a given age group and between the age groups studied.

Electron micrographs have shown that the superficial cell is the largest cell in the urothelium and Table 1 and text Fig. 1 show that its profile area is nearly two and a half times that of the intermediate cell. The intermediate cell is marginally larger than the basal cell. The nuclei of the superficial cells, however, is only about one and a half times the size of that of the intermediate cells (Table 2 and text Fig. 2). The nuclei of the intermediate and basal cells are not strikingly different from each other in size.
The nucleo-cytoplasmic ratio decreases from about 0.725 in the basal cell to about 0.561 in the intermediate cell and 0.285 in the superficial cell (Table 3 and text Fig. 3).

As regards the volume densities of cellular organelles, in each cell type, the values were calculated for (a) mitochondria, (b) polymorphic bodies, (c) the group of membranous structures comprising the Golgi complexes, fusiform and round vesicles, and (d) rough endoplasmic reticulum. The mean volume densities of mitochondria in the superficial and intermediate cells were nearly the same and the value in the basal cell was also not far different (Table 5 and text Fig. 5).

A different picture emerged when the volume density of polymorphic bodies was determined: the highest value was obtained in the superficial cell and the least in the basal cell with the intermediate cell value somewhere in between but fairly close to that in the basal cell (Table 7 and text Fig. 7). The data indicate that the volume density of polymorphic bodies which most probably are secondary lysosomes, or residual bodies, is markedly high in the superficial cells of the urothelium even in the 10 days old rats. Likewise, the highest volume density of Golgi apparatus, fusiform and round vesicles was also found to occur in the superficial cell (Table 4 and text Fig. 4). The volume density factor for the rough endoplasmic reticulum was the highest in intermediate cells, and the lowest in the superficial cells (Table 6 and text Fig. 6); this was the only cellular organelle in the urothelium of 10 days old rats to show a higher incidence in intermediate and basal cells than in the superficial cell. Table 8 and text Fig. 8 reflect the richness of the organelle-content in the superficial cell where, as a consequence, the volume fraction occupied by cytoplasmic matrix is the lowest in the urothelium.
Adult rats (10 months old)

Three layers of cells were invariably observed in the urothelium of 10 month old rats. The extracellular space between the urothelial cells was narrow as in the young rats and the extent of interdigitation of the plasma membranes of cells in different layers was similar to that in the previous group. Unlike in the younger specimens, the superficial cells appeared less electron dense overall, and stood out in this respect from the underlying layers of cells (Fig. 11).

Cell junctions and contacts. The junctional complexes between the lateral borders of superficial cells near the luminal surface appeared to be no different from those in the younger animals. However, desmosomes seemed to occur with greater frequency than before (Figs. 17, 18). In addition, the hemi-desmosomes along the basal cell-stromal interface were obviously better developed than in the younger specimens and there were also far more of them per unit length of the border (Fig. 12).

The luminal surface in TEM and SEM. The luminal membrane had the characteristic scalloped appearance (Figs. 11, 15) as in the young rat and the asymmetric membrane of its angular regions was 12 nm thick, as described before. There was, however, a noticeable increase in the number of fusiform vesicles compared to that in the 10 days old rat, and more will be said of this later.

In low power scanning EM, the superficial cells appeared as large 4 to 7 sided cells (Fig. 13). The cells appeared to be more uniform in size than in the young urothelium. In high power micrographs, the luminal surface showed a somewhat complicated pattern of
folds or shallow microridges (Fig. 14). These microridges were also more extensive than before. Another prominent feature was the presence of a large number of craters (many more than in the young) distributed all over the surface.

Superficial cells. The most notable constituent of the apical portion of the cell was the numerous fusiform vesicles oriented in both horizontal and vertical directions in relation to the luminal surface (Fig. 15). The asymmetric nature of the limiting membrane of these vesicles with the thicker lamina lining the vesicle lumen was easily observed in high power pictures. Also seen in the superficial cell cytoplasm were a few round vesicles and others of irregular shapes and sizes limited by symmetrical unit membranes. Apart from the abundance of the fusiform vesicles, a closer examination showed that those close to or adjoining the luminal plasma membrane were the ones oriented vertically, whereas, those further below were more horizontally oriented. The observation of a large number of craters observed in scanning micrographs corresponds to the openings on to the cell surface of the large number of vertically oriented fusiform vesicles.

Golgi complexes were generally more extensive than those of the younger specimens. Stacks of cisternae, some of them dilated, were one of the main components of the complexes; several AUM-limited cisternae were also noticeable in the area (Fig. 16). Besides cisternal profiles, a number of small vesicles measuring 80-100 nm in diameter formed the other main component of the Golgi complexes. Many of these displayed bristly coats so characteristic of entities referred to in the literature as coated vesicles.

Another notable feature of the superficial cell was the large
number of polymorphic bodies with irregular shape and differing sizes, measuring 0.5 µm to 1.6 µm in average diameter. Some of them contained, what appeared to be, fusiform vesicles; others enclosed heterogeneous material and their matrix was more electron dense than the surrounding cytoplasm. Polymorphic bodies which seemed to consist predominantly of lipid material (Fig. 19) were also met with occasionally.

Mitochondria were abundant but profiles of rough endoplasmic reticulum were scarce. The cytoplasmic matrix contained mainly microfibril and free ribosomes (Fig. 15); neither of these was there in prolific quantities and microfibrils were probably fewer than in the previous age group.

The nuclear envelope was smooth and the nuclei and nucleoli were, as in the previous age group, except that the chromatin was more diffuse and uniformly distributed.

 Intermediate cells. The Golgi complexes were situated in the apical regions of the cytoplasm and consisted of small cisternal profiles and small vesicles. No AUM-limited cisternal or vesicular profiles were detected in the Golgi complexes of these cells (Fig. 17). Polymorphic bodies were frequently noticed in the cell and so were profiles of rough endoplasmic reticulum. There was no dearth of mitochondria (Figs. 11, 18). In the nucleus, there was a noticeable layer of relatively condensed chromatin along the nuclear envelope as seen in Fig. 17.

 Basal cells. There was practically no difference in the ultrastructure of the basal cells of this age group from that in the younger stage: however, there seemed to be more mitochondria than before.
Additionally, a few polymorphic bodies of varying sizes were noted. A relatively large proportion of the nuclear chromatin of this cell was heterochromatic as in the basal cells of the previous age group.

The lamina densa was generally thicker and more electron dense than that of the young rats. It measured about 50 nm except in the regions opposite the hemi-desmosomes where the thickness increased to 75 nm (Fig. 12). The lamina lucida was also wider than in the previous group and measured about 40 nm but it narrowed to about 10 nm in the region of the hemi-desmosomes.

In the connective tissue below, the collagen fibres seemed to be much more abundant (Figs. 11, 12) than in the 10 days old animals.

**Morphometric analysis of the urothelial cells.** The mean profile areas of the cells and the nuclei of the three cell types are not very different from those obtained in the 10 days old rats (Tables 1 and 2 and text Figs. 1 and 2). Likewise, the nucleo-cytoplasmic ratio also decreases from 0.70 in the basal cell to 0.53 in the intermediate cell and 0.23 in the superficial cell (Table 3 and text Fig. 3).

The mitochondria occupied a greater percentage of the cytoplasm in cells of all layers than in the previous group. The mean volume densities of this organelle are presented in Table 5 and text Fig. 5 where it may be noted that the increase is most pronounced in the intermediate cells which now become the cells with the highest percentage of their cytoplasmic volume occupied by mitochondria in the urothelium of the adult rat.

The mean volume density of Golgi complexes and the vesicles was found to be significantly higher than in the superficial cells of the young specimens. However, the mean values of these profiles in the
intermediate and basal cells, were not significantly different from those of the young group (Table 4 and text Fig. 4). Also, the volume density of endoplasmic reticulum was significantly higher than in the superficial cells of the young specimens, but the mean values of this organelle in the intermediate and basal cells were not significantly different from those of the young group (Table 6 and text Fig. 6).

Data presented in Table 7 and text Fig. 7 show clearly the remarkably high volume densities of the polymorphic bodies in the cytoplasm of both superficial and intermediate cells. The values are roughly over 2½ times higher than in the young specimens. It may also be noted that the increase is insignificant in the case of the basal cells.

The mean volume density and cytoplasmic matrix in the superficial cells is significantly less than that of the young specimens. However, the mean values in the intermediate and basal cells were not significantly different from those of the young group (Table 8 and text Fig. 8).

**Aged rats (20 months old)**

Three layers of cells could be clearly demonstrated in the urothelium of the distended bladder (Fig. 20), as in the adult, and in the major part of the urothelium in the young. The extracellular space in the urothelium continued to be narrow and the extent of interdigitation between the cells in different layers was also as in the bladder of the adult animals. The overall appearance of the urothelium in TEM micrographs was therefore much like that of the adult animal except for the sporadic occurrence of small, blunt microvilli-like processes at the luminal surface (Figs. 22, 23). The electron density of the superficial cells was generally lower than that of the underlying cells (Figs. 20, 32).
Cell junctions and cell contacts. A higher power electron micrograph of the junctional complex between two superficial cells close to the lumen is shown in Fig. 21. The structure and disposition of the various types of junctions are apparently the same as in the adult described earlier. Desmosomes were observed almost as frequently as in the urothelium of the adult, and so were hemi-desmosomes along the basal border facing the basal lamina (Figs. 31, 33).

The luminal surface in TEM and SEM. The scalloped nature of the luminal surface continued to predominate in the 20 month old rat (Figs. 20, 32) and there was also no dearth of fusiform vesicles in the apical cytoplasm close to the plasma membrane. For the most part, therefore, the luminal surface showed the scalloped appearance as in the previous group, but occasionally, there were present non-angular regions with a wavy contour. These regions appeared flexible so as to form microvilli-like structures as shown in Figs. 22 and 23. The microvilli were small (about 0.28 μm high) and their free ends were wider than the base. The symmetrical structure of the luminal membrane at these sites is clear in Fig. 22.

SEM of the luminal surface usually showed tightly adherent cells with polygonal profiles resembling a pavement (Fig. 24) as the urothelium of adult rats described before. Such pictures also showed an intricate pattern of folding of the cell surface. The network of ridges are seen even more clearly in higher power pictures such as that shown in Fig. 25, but amidst these were occasionally found short microvilli. An area of surface where several microvilli were noticeable is presented in Fig. 26: such instances were rare but nevertheless present in the urothelium of this age group.
Superficial cells. In the majority of electron micrographs examined, and especially in low power pictures, the apical portion of the cell seemed to differ very little from that of the adult tissue. Fusiform and elongated vesicles were abundant and oriented in vertical and horizontal directions (Figs. 20, 32, 33). The asymmetrical nature of the luminal membrane, about 12 nm thick, as well as the limiting membrane of the more fusiform looking vesicles is also clear in these micrographs. Micrographs were, however, obtained of apical regions which contained more elongated vesicles limited by a symmetrical unit membrane, about 10 nm thick (Fig. 22). The morphological characteristics of the luminal surface along such regions were also different. Instead of angular profiles, the luminal membrane appeared relatively smooth and symmetrical with blunt microvilli-like structures (Figs. 22, 23) as mentioned before.

The Golgi complex was usually well developed in the superficial cells (Fig. 27) and the area abounded in asymmetrical unit membrane-limited vesicles. However, there were also present several rounded vesicles limited by symmetrical membrane. It may further be noted that most of the AUM-limited vesicles are situated in the peripheral area of the Golgi zone, and toward the apical surface of the cell, whereas the round vesicles are in the central area of the Golgi apparatus close to the cisternal stacks. Coated vesicles were present but scarce. Another example of the Golgi apparatus and the dispositions of fusiform vesicles is shown in Fig. 30, where profiles of rough endoplasmic reticulum are also present. It may be noted again that most of the AUM-limited fusiform vesicles in this instance also are located away from the Golgi apparatus and toward the apical region of the cell. Traces of elongated, symmetrical membrane-limited vesicles
are discernible (arrows) very close to the Golgi apparatus.

Polymorphic bodies, and microfibril bundles were observed as in the superficial cells of the previous group. Free ribosomes were present but not in abundance, and among the mitochondria, there were more instances of degenerating forms than in the previous age groups. The volume density measurements do not reflect this since they refer only to the number per unit cell volume and included both normal and degenerating mitochondria. The ultrastructural morphology of the nucleus and nucleolus was as described before in adult rats.

**Intermediate cells.** Unlike in previous age groups, the intermediate cells of the present group often contained many fusiform and elongated vesicles in their apical cytoplasm (Figs. 20, 32, 33). In this respect, the intermediate cells bore a close resemblance to the superficial cell and at times the similarity was very striking (Fig. 33). High power examination also showed that several vesicles were limited by symmetrical membrane, especially those which were more elongated rather than fusiform. In certain cells such as that presented in Fig. 28, the Golgi apparatus was fairly well developed but showed no trace of asymmetric membrane in the area. Elongated vesicular profiles present were limited by symmetric unit membrane. Also present in the area were small vesicles of the coated variety.

Other organelles such as mitochondria, endoplasmic reticulum, polymorphic bodies and free ribosomes were as in the intermediate cells of the 10 month old rats already described. The ultrastructural morphology of the nucleus (Fig. 28) was also similar to what was described before (Fig. 17) in the intermediate cells of adult rats.
Basal cells. There were not any obviously striking ultrastructural features to distinguish the basal cell of this age group from that of the preceding groups examined, particularly the 10 month old specimens. The cells were small with smooth, more or less rounded, nuclei containing much of the chromatin in a condensed form.

In the cytoplasm, there were polymorphic bodies and abundant free ribosomes. The rough endoplasmic reticulum tended to be organised as elongated cisternae rather than scattered vesicles in the vicinity of the nucleus and extended to the site of the Golgi apparatus which, as usual in basal cells, was situated on the basal side of the cell (Fig. 29). The Golgi apparatus appeared as in basal cells of previous age groups. Apart from the closely packed saccules, the main vesicular component of the Golgi apparatus was of the coated variety. Fusiform vesicles with asymmetrical membrane or other large vesicles with symmetrical membranes were never met with in these cells either in the Golgi zone or elsewhere.

The basal lamina (31, 60, 33, 62) was similar to that of the adult with the lamina densa measuring about 55 nm in thickness generally, but widening to about 70 nm in the regions facing the hemi-desmosomes. The lamina lucida measured about 30 nm in thickness except in the region of the hemi-desmosomes where it was much narrower, being only about 10 nm thick.

In the lamina propria, collagen fibres appeared to be even more numerous than in the 10 months old rats (Fig. 33).

Morphometric analysis of the urothelial cells. As shown in Table 1 and text Fig. 1, there are no significant differences in the profile areas of the three types of cells in the urothelium of the 20 months old rats when compared with those of the younger age groups. The
profile areas of the nuclei in each of the cell types are also not significantly different from that observed in the other two age groups (Table 2 and text Fig. 2); the nucleo-cytoplasmic ratios of the three cell types therefore continue unchanged in this group (Table 3 and text Fig. 3).

As for the volume densities of mitochondria, and polymorphic bodies, the values remain practically unchanged (Tables 5 and 7 and text Figs. 5 and 7) when compared with those found in the preceding age group. The values of endoplasmic reticulum remain unchanged for the intermediate and basal cells, but significantly less than that of the 10 months old for the superficial cells (Table 6 and text Fig. 6).

Unlike the above mentioned values, the volume density of Golgi complex, and the various vesicles derived and associated with it, is higher in the superficial cells of the 20 month rat over that of the 10 month old specimens (Table 4 and text Fig. 4). The increase is even more significant in the intermediate cells. The table also shows that the highest volume density of the Golgi group of organelles occurs in the superficial cells of the 20 month old rats. Also, the highest value for this group of organelles in intermediate cells is in this age group. As far as the basal cells are concerned, the relative volume of Golgi component seems to remain unchanged from the young urothelium to the old.

The mean volume densities of the cytoplasmic matrix of the superficial and intermediate cells are significantly lower than in the previous group. But the values do not differ significantly in the basal cells (Table 8 and text Fig. 8).
Table (1) Cell area of the normal rat urothelium in \( \mu m^2 \).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>10 days old</th>
<th>10 months old</th>
<th>20 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>281.57 ± 38.56</td>
<td>305.42 ± 49.85</td>
<td>327.05 ± 54.95</td>
</tr>
<tr>
<td>Intermediate</td>
<td>112.28 ± 19.24</td>
<td>117.16 ± 18.17</td>
<td>124.15 ± 21.55</td>
</tr>
<tr>
<td>Basal</td>
<td>77.35 ± 17.23</td>
<td>72.13 ± 18.26</td>
<td>79.50 ± 20.33</td>
</tr>
</tbody>
</table>

Table (2) Nuclear area of normal rat urothelium in \( \mu m^2 \)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>10 days old</th>
<th>10 months old</th>
<th>20 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>62.50 ± 11.29</td>
<td>57.50 ± 12.58</td>
<td>65.30 ± 14.08</td>
</tr>
<tr>
<td>Intermediate</td>
<td>40.33 ± 8.86</td>
<td>40.78 ± 8.43</td>
<td>42.50 ± 9.65</td>
</tr>
<tr>
<td>Basal</td>
<td>32.50 ± 4.65</td>
<td>29.69 ± 3.43</td>
<td>32.50 ± 4.65</td>
</tr>
</tbody>
</table>
Table (3)  Nuclear-cytoplasmic ratio of normal rat urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>10 days old</th>
<th>10 months old</th>
<th>20 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.285 ± 0.062</td>
<td>0.232 ± 0.048</td>
<td>0.249 ± 0.051</td>
</tr>
<tr>
<td></td>
<td>(1:3.51)</td>
<td>(1:4.31)</td>
<td>(1:4.02)</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.561 ± 0.088</td>
<td>0.534 ± 0.085</td>
<td>0.521 ± 0.080</td>
</tr>
<tr>
<td></td>
<td>(1:1.78)</td>
<td>(1:1.87)</td>
<td>(1:1.92)</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Basal</td>
<td>0.725 ± 0.136</td>
<td>0.700 ± 0.131</td>
<td>0.691 ± 0.128</td>
</tr>
<tr>
<td></td>
<td>(1:1.38)</td>
<td>(1:1.43)</td>
<td>(1:1.45)</td>
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<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
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</tr>
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</table>

Table (4)  Volume density of Golgi body and vesicles in normal rat urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>10 days old</th>
<th>10 months old</th>
<th>20 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.158 ± 0.011</td>
<td>0.198 ± 0.018</td>
<td>0.316 ± 0.046</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.05</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.050 ± 0.008</td>
<td>0.053 ± 0.007</td>
<td>0.124 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Basal</td>
<td>0.016 ± 0.004</td>
<td>0.024 ± 0.005</td>
<td>0.023 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
### Table (5) Volume density of mitochondria in normal rat urothelium

<table>
<thead>
<tr>
<th>Cell type</th>
<th>10 days old</th>
<th>10 months old</th>
<th>20 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.077 ± 0.007</td>
<td>0.125 ± 0.013</td>
<td>0.117 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.080 ± 0.005</td>
<td>0.157 ± 0.016</td>
<td>0.175 ± 0.018</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.068 ± 0.009</td>
<td>0.105 ± 0.009</td>
<td>0.118 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.001</td>
<td></td>
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</tr>
</tbody>
</table>

**Note:** N.S. indicates non-significant difference.

### Table (6) Volume density of endoplasmic reticulum in normal rat urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>10 days old</th>
<th>10 months old</th>
<th>20 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.016 ± 0.004</td>
<td>0.034 ± 0.005</td>
<td>0.025 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.043 ± 0.007</td>
<td>0.054 ± 0.007</td>
<td>0.046 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>0.038 ± 0.006</td>
<td>0.043 ± 0.005</td>
<td>0.038 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** N.S. indicates non-significant difference.
Table (7)  Volume density of polymorphic bodies in normal rat urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>10 days old</th>
<th>10 months old</th>
<th>20 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.039 ± 0.007</td>
<td>0.093 ± 0.019</td>
<td>0.099 ± 0.022</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.014 ± 0.004</td>
<td>0.032 ± 0.007</td>
<td>0.046 ± 0.007</td>
</tr>
<tr>
<td>Basal</td>
<td>0.009 ± 0.002</td>
<td>0.013 ± 0.003</td>
<td>0.016 ± 0.003</td>
</tr>
</tbody>
</table>

Table (8)  Volume density of cytoplasmic matrix in normal rat urothelium

<table>
<thead>
<tr>
<th>Superficial</th>
<th>10 days old</th>
<th>10 months old</th>
<th>20 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.508 ± 0.030</td>
<td>0.388 ± 0.016</td>
<td>0.315 ± 0.012</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.581 ± 0.024</td>
<td>0.607 ± 0.037</td>
<td>0.461 ± 0.021</td>
</tr>
<tr>
<td>Basal</td>
<td>0.678 ± 0.026</td>
<td>0.657 ± 0.037</td>
<td>0.684 ± 0.016</td>
</tr>
</tbody>
</table>
TEXT FIG. (1) THE UROTHELIAL CELL AREA IN NORMAL RATS.

TEXT FIG. (2) THE NUCLEAR AREA OF THE UROTHELIAL CELL IN NORMAL RATS.

TEXT FIG. (3) THE NUCLEAR CYTOPLASMIC RATIO IN NORMAL RAT UROTHELUM.

TEXT FIG. (4) THE VOLUME DENSITY OF GOLGI BODIES AND VESICLES IN NORMAL RATS.
TEXT FIG. (5) THE VOLUME DENSITY OF MITOCHONDRIA IN NORMAL RATS.

TEXT FIG. (6) THE VOLUME DENSITY OF ENDOPLECTIC RETICULUM IN NORMAL RATS.

TEXT FIG. (7) THE VOLUME DENSITY OF POLYMORPHIC BODIES IN NORMAL RATS.

TEXT FIG. (8) THE VOLUME DENSITY OF CYTOPLASMIC MATRIX IN NORMAL RATS.
DISCUSSION

Examination of the urothelium from dilated bladders of the 10 day old rats show that much of the urothelium is 3 layered, but there are present stretches, which are only 2 layers thick. During early development of the bladder of albino rats, it is known that the urothelium passes through uni- and bilaminar conditions and the second layer, formed between the sixteenth and nineteenth days of gestation, comes to possess differentiated features like angular luminal membrane, fusiform vesicles and Golgi cisternae with plaques of thickened membrane (Firth and Hicks, 1972). According to these workers, this, the first generation superficial cells, desquamate during the perinatal period and a new superficial layer is generated by mitosis of the basal cells during the second week after birth. The new or second generation superficial cells show fusiform vesicles and an angular profile, but Firth and Hicks (1972) reported that the 3 layered condition characteristic of mature urothelium becomes established only by the end of the third week after birth. My observations show that, in the Wistar rats the urothelium is generally well developed by 10 days after birth since by this time, much of the urothelium consists of 3 cell layers. The present observations (Fig. 4) confirm the findings of previous workers that in the regions where only 2 cell layers are present, the superficial cells - presumably the first generation superficial cells - are clearly differentiated with an angular surface profile and several fusiform vesicles. However, in regions where 3 layers of cells occur, it is only the superficial cell that is differentiated; in no instance did I come across an intermediate cell that also showed signs of differentiation except in the rare instances mentioned earlier where the urothelium
was 4-cell thick and the intermediate cell immediately below the superficial cell exhibited some signs of differentiation. As a rule, therefore, morphological differentiation appears to be restricted to the most superficial layer of cells, irrespective of whether the urothelium is 2 or 3 layers thick. An exception to this will be mentioned later. Along with the establishment of the 3 layered condition, a series of intercellular junctions are observable at the lateral borders of the superficial cells near the luminal surface. These correspond to the combinations of cell surface specializations referred to in the literature as junctional complexes or terminal bars. The outermost of these is the so-called tight junction or zonula occludens which in the 10 days old rat is fully formed as far as can be judged by transmission electron microscope study of thin sections. The ultrastructural organisation of the intermediate junction (zonula adherens) and the desmosome (macula adherens) is also quite similar to those described in the literature in a variety of adult mammalian epithelia. Junctional complexes, as above, have also been noticed during this study between the first generation superficial cells, i.e., the superficial cells in the 2-cell thick stretches of urothelium. It would therefore seem that the development of the junctional complex along the lateral cell border near the lumen is an essential accompaniment of the differentiated state of the superficial cell which distinguishes it from those of the underlying layers.

In the 3-cell thick regions, the superficial cells show considerable variation in size. Many of them are markedly larger than the intermediate cells, but others are not. Also the first generation superficial cells in the 2-cell thick regions are usually not larger than the underlying cells.
My findings, together with those of Firth and Hicks (1972), establish that the 3-layered condition of the urothelium comes to be largely laid down in rats during the second and third week after birth. Phillips and Davies (1980) have reported that the 3-layered condition is constant in female mice of the age range 2-33 months, but they did not examine younger animals to establish when this condition first became apparent.

In the 10 month old or adult rats that I examined, the urothelium was generally 3-cell thick and the superficial cells were larger and uniformly so than in the 10 day old rats. Of the three age groups studied the uniformity in size of the superficial cells was best seen in SEM pictures of the adults.

The nature and dimension of the extra cellular space remained more or less the same in all ages and especially in the adult and aged specimens. More desmosomes seemed to occur between urothelial cells in the adult and old rats compared with the 10 day old specimens. There were no gross morphological changes in the basal lamina with advancing age, although in the adult stage and in later life the lamina densa was found to be thicker than in the very young.

The superficial cell is undoubtedly the largest of the urothelial cells in all the age groups and the morphometric analyses show that the relative areas occupied by the superficial, intermediate and basal cells in urothelia are practically unchanged throughout the life span. Likewise, the nucleo-cytoplasmic ratio in each of the cell types remains more or less the same throughout the life span of the animal. But the volume density of the Golgi apparatus and associated or derived vesicles shows a progressive
increase with age; the increase is especially prominent through the 10 month old to the 20 month old animals. The volume density of Golgi and associated vesicles of the intermediate cells does not change from the young to the adult but a significant increase in its value occurs between the adult and the later age group studied as in the case of the superficial cells. The volume fraction occupied by Golgi apparatus and the vesicular components is always highest as a rule in the superficial cells of the urothelium (Table 4) at any age. The above observations show that the Golgi components become more and more extensive with age in both superficial and intermediate cells reaching a peak in both the cell types in the oldest specimens studied. The volume density of Golgi and associated vesicles of the basal cells is unaltered throughout the life span. More about the Golgi apparatus and its functions will be said later.

The mitochondrial volume fraction is much the same in all cell types of the young rat, and taken as a whole, these values are significantly lower than in the older specimens. It may be that the metabolic activities of the urothelium as a whole in the 10 day rats is significantly lower than in the later stages.

Another notable observation that has emerged from the morphometric analyses relates to the rough endoplasmic reticulum. Although the volume fraction of this organelle in the superficial cells of the adult and later stage increases significantly over that in the young, it is interesting to note that the highest volume density fraction of endoplasmic reticulum is not in the superficial cells but in the intermediate cells in all age groups. It should, however, be borne in mind that the total amount of endoplasmic reticulum is still likely to be greater in the superficial cell because of the relatively larger volume of this cell. The function of the rough endoplasmic
reticulum has been well established; at the present time, a well
developed reticulum is known to correlate with synthesis of
secretory and other proteins which require to be transported from
the site of synthesis to locations elsewhere in the cell, as for
example lysosomes, cell coat material and membrane proteins.
Reports of previous workers (for instance, Firth and Hicks, 1973)
would tend to suggest that rough endoplasmic reticulum is hardly
present in rat urothelial cells. It is possible that they con-
veyed this impression on the basis of examining a few isolated
micrographs. My data show, however, that this membrane system
constitutes a volume fraction not all that insignificant even in
the basal cells of the 10 days old rats. In the light of this
finding, it is of interest to note that in rabbits, bladder uro-
thelium has been shown to secrete a glycosaminoglycan (Parsons
et al., 1980), the presence of which on the cell surface is be-
lieved to reduce the ability of bacteria to adhere to the cells.
Another involvement of the endoplasmic reticulum may be in the
synthesis of the large quantities of β-glucuronidase which has
been reported to be present in both rat and human urothelia
(Connolly et al., 1973).

A membrane-limited body which has been described prominently
in many accounts of mammalian urothelial cells is the polymorphic
body. This includes large spherical vesicles of various sizes
with heterogeneous content and which bear morphological similarities
to entities described in the electron microscope literature as
multivesicular bodies, secondary lysosomes and autophagic bodies.
My data confirm that among the three cell types, polymorphic
bodies are most abundant in the superficial cells of adults and
old rats. Furthermore, the data also point to a remarkable and steady increase in the quantity of the polymorphic bodies in the superficial and intermediate cells as the animal ages. This contrasts with the low, and more or less stationary, numbers of these bodies in the basal cells of the urothelium throughout the life span of the animal.

The view emerging from observations cited so far, that the basal cells represent a stable and ultrastructurally unchanging cell population in the urothelium throughout the life span is further reinforced by the volume density values of cytoplasmic matrix. On the other hand, the superficial cells show a progressive decline in cytoplasmic matrix volume density from the 10 day old, through the 10 month old to the 20 month old specimens. This is no doubt, a reflection of the increasing content of various organelles in these cells and a consequent decrease of cytoplasmic matrix. Taken as a whole, the quantitative data point to the superficial cells as the most dynamic cell population in the urothelium.

The most important change that has been noted in the bladder urothelium in relation to age pertains to the superficial cells. There is a striking difference in the surface topography of the adult urothelium from the simple pattern of shallow microridges observed in the younger stage; this is best visualized in the scanning electron micrographs in Figs. 7 and 14. The difference appears to be due mainly to the large number of fusiform or discoidal vesicles oriented perpendicularly to and in communication with the luminal surface. Transmission electron micrographs further show that in the adult the apical cytoplasm is filled with fusiform vesicles, many of which are also oriented parallel
to the surface and others oriented randomly. As stated before, it is well establised by other workers (Hicks, 1966b; Koss, 1969) that the fusiform vesicles bounded by asymmetrical unit membrane originate in the Golgi apparatus of maturing urothelial cells and that these vesicles eventually contribute their asymmetric membrane to the luminal surface. This contribution of the Golgi apparatus is now widely regarded as its most specialised function in mammalian urothelium. Clearly the organelle is at the peak of its activity in the 10 month old animals resulting, if anything, in a surfeit of asymmetric unit membranes. The Golgi apparatus has been credited with a multiplicity of functions in secretory and non-secretory cells (Tartakoff, 1980), but in the adult urothelium, there is little doubt that this organelle is mainly involved in the assembly of the specialised asymmetrical plasma membranes, the fusiform vesicles being the form in which the asymmetric membrane is produced and transported. As a result the luminal surface shows a predominantly angular conformation and the adult bladder is presumably well endowed with a reserve supply of the specialised membrane (in the form of fusiform vesicles) for insertion into the surface and thus enable maximal stretching of the superficial cells during bladder distension.

A careful study of the Golgi apparatus, Golgi-derived vesicles and the luminal plasma membrane has revealed certain interesting features in the older animals. The Golgi complex in the 20 month old rats was more extensive than in the adult and there was no dearth of asymmetrical unit membrane-bounded fusiform vesicles in the area. There were also present, however, several more or less spherical and elongated vesicles bounded by symmetrical membrane.
The more interesting point relates to the distribution of the vesicular components in the area. The initial impression gained from a preliminary study and subsequently confirmed by examining a large number of electron micrographs is that the elongated vesicles are located close to the cisternal stacks of the Golgi apparatus. This is not to say that an occasional fusiform vesicle was not found in the area but that the fusiform vesicles tended to be located more in the periphery of the Golgi zone. A simple interpretation of the varying dispositions of the two populations of Golgi-derived vesicles would be that the asymmetric membrane-limited, fusiform vesicles were processed earlier than the symmetrical membrane-limited, spherical vesicles. An implication of this is that, although the Golgi apparatus continues to be functionally active in the older specimens, the nature of the membrane emerging from the Golgi apparatus gradually changes. It is significant that the first appearance of some stretches of smooth, flexible luminal plasma membrane in the urothelium of 20 month old rats parallels the occurrence of symmetrical membrane-limited vesicles in the Golgi zone of the luminal cells. This tendency that has come to light as the animal ages, that is, the production of symmetrical membrane-limited vesicles instead of asymmetric membrane-limited vesicles, in both superficial and intermediate cells, may involve a switch in gene expression affecting plasma membrane biosynthesis. It may be that in animals older than 20 months, the proportion of symmetrical membrane at the luminal surface would become even greater, but it was not possible for me to extend this study owing to the difficulty of obtaining rats older than 20 months. The hypothesis suggested above, therefore remains to be confirmed in older animals or those
maintained to the natural end of their lives. It is of interest at this juncture to recall that the luminal membrane is symmetrical in structure during gestation (Firth and Hicks, 1972) and that the switch to asymmetrical structure occurs at about the time of birth.

The volume density of polymorphic bodies in the superficial cells is as high in the 20 month specimens as in the 10 month specimens. These bodies are very heterogeneous in size and content, and they are generally accepted to be autophagic bodies or cytolytosomes which serve as waste bins for old or surplus organelles and also unused asymmetric membranes (Hicks, 1965; Koss, 1969; Hicks, 1973; Jacob et al., 1978). The necessary production of lysosomes, containing several hydrolytic enzymes, antecedent to the formation of autophagic bodies involves, as is well known, the Golgi apparatus and this illustrates another functional role of the rough endoplasmic reticulum and the Golgi apparatus in the urothelial cell in addition to the production of cytomembranes. The continued high incidence of polymorphic bodies in the aged animals indicates continued production of lysosomes - a Golgi function which therefore appears to proceed unimpaired in the old animal. If this hypothesis is correct, one might expect to find in the superficial cells of animals older than those used in this study, polymorphic bodies containing surplus symmetrical membranes.

Yet another observation which merits discussion is the state of differentiation of the intermediate cells during the life span of the rat. It was pointed out earlier in this discussion that in the young and adult, the intermediate cells as a rule show no great manifestations of differentiation which are so conspicuous in the superficial cells of all ages. Therefore, the picture of the
intermediate cells in the 20 month old urothelium with many fusiform and elongated vesicles in the apical regions is somewhat unique. The intermediate cells at this age may hence be said to exhibit a generally higher degree of differentiation than that attained by corresponding cells in the two previous age groups. There have been sporadic references in previous publications (cf. Koss, 1969) to the initiation of AUM production in intermediate cells of rats, but as far as my survey of the literature goes, there are no reports of intermediate cells exhibiting consistently overt features of differentiation as I have described in the 20 months old animals. The intermediate cells of the old age group, more than any other group, exemplify what has been known from other, especially experimental, studies that this layer of cells is indeed the precursor of the superficial layer. It is nevertheless, surprising that the fusiform and elongated vesicles were hardly ever seen to be actually incorporated into or in continuity with the apical plasma membrane of the intermediate cells.

In this context, it is interesting to recall an unusual condition which I have described in the 10 day rats. It was stated, how the intermediate cell immediately adjoining the superficial cell in rare instances of 4-cell thick urothelium, resembled the superficial cell to the extent of an AUM-limited vesicle appearing in continuity with the apical plasma membrane of the cell. In the light of the above discussion, regarding the state of differentiation of intermediate cells in general during the major life span of the animal, it would seem likely that the upper of the two layers of intermediate cells is in reality, a second generation superficial cell held in abeyance by a rare failure of the first generation superficial cell to desquamate during the perinatal period.
CHAPTER II

ULTRASTRUCTURE OF NORMAL HUMAN BLADDER UROTHELIIUM

AND AGE RELATED CHANGES

Introduction

There have been few descriptions of the ultrastructure of human bladder urothelium in the past and several reasons can be advanced for this. One reason for this is undoubtedly the difficulty of obtaining material. Immediate removal of organs from young healthy individuals who have died from irreversible head injury from automobile and other accidents could provide one answer to the problem, but as far as I am aware, such samples have not been used for electron microscopic investigations of the bladder. Another source of specimens has been cystectomy specimens and necropsy bladders (Firth and Hicks, 1973; Marsh et al., 1974; Newman and Hicks, 1977; Davies and Hunt, 1981). Merk et al. (1977), have used specimens of human bladder obtained at autopsy from post-natal infants which, according to these workers, had grossly normal appearing urothelium. My attempts to use routine necropsy material obtained from the Sick Children's Hospital, Edinburgh, proved quite unsatisfactory. Necropsy bladders of abortuses and children were obtained in an effort to examine the ultrastructure of the very young but the processed material turned out to be unsuitable in every instance since the urothelium appeared badly damaged by autolytic changes. The problems to be overcome are the effects of anoxia and post-mortem changes. The time interval between tissue death and tissue removal and fixation is of far more critical importance for ultrastructural studies than for
routine histological examination.

The quality of structural preservation of the tissue as a whole required for ultrastructural studies can only be met by rapid fixation of fresh tissue and the procedures developed to minimise structural distortion of the original state are much more stringent than those usually employed to process material for examination at the light microscope level. The extractability of tissue constituents such as lipids, proteins, lipoproteins and carbohydrates at the time of fixation and during subsequent processing is another problem that affects the quality of the tissue in studies of cellular ultrastructure and this again calls for special fixation procedures such as double fixation that are not usually employed in routine light microscope studies of tissue structure. Also other factors such as temperature, pH, ionic strength, ionic composition and osmolality of the fixative are more critical in determining the quality of fixation at the level of the electron microscope.

In situ or in vivo fixation of tissue as practised in the case of laboratory animals is far superior to in vitro fixation but in the case of human tissues, fixation of fresh biopsy or surgical material offers the best available means for avoiding anoxic insult and autolytic changes. Many of the investigators of human bladder urothelium have used biopsy specimens obtained at cystoscopy but the nature of standard cystoscopy procedures involving filling the bladder with sterile distilled water (Merk et al., 1977; Price et al., 1980; Jacobs et al., 1981) can induce artifacts. Very few of the investigators have, however, mentioned what fluid was instilled into the bladder which might have been distilled water, as mentioned above, or a glycine solution with
a low osmotic pressure. Osmotically this fluid compares very unfavourably to urine (Tannenbaum, 1979). Others have tried to overcome the problem of artifacts arising from unphysiological instillation fluids by basing their observations exclusively on non-cystoscoped urothelium; urological specimens that contained urothelium were taken in these instances at the time of surgery, as for instance during prostatectomy (Firth and Hicks, 1973; Tannenbaum et al., 1978; Alroy et al., 1981). Since patients with obstructive diseases of the urinary tract may not be entirely suitable for the study of normal bladder urothelium (see also later), Haynes et al. (1975) obtained urothelium from boys undergoing surgery for congenital lesions of the lower urethra in whom there was no evidence of other urological abnormality or urinary tract infection. Likewise there was one study (Hoyes et al., 1972) when bladders were examined from human foetuses obtained after their removal from the uterus at hysterotomy.

By far the greatest stumbling block to systematic studies with the transmission electron microscope of human tissues is the lack of availability of entirely normal, non-diseased material. In some of the earliest descriptions of adult human urinary bladder epithelium such as those of Kashiwai et al. (1963), the authors did not give information on the source of the tissue but Battifora et al. (1964) stated that the endoscopic biopsies were obtained from patients with benign hyperplasia of the prostate. On the other hand, Fulker et al. (1971) obtained normal bladder epithelium from non-tumour bearing areas of patients with bladder cancer by transurethral endoscopic resection. In most of the subsequent TEM as well as SEM investigations
also, normal urothelium has meant urothelium which at cystoscopic examination had normal appearance and had been obtained from patients with bladder cancer or who had urothelial cancer elsewhere in the urinary tract (for example, Fulker et al., 1971; Kinoshita, 1972; Kjaer et al., 1976; Merk et al., 1977; Jacob et al., 1978; Price et al., 1980). Urologists are understandably reluctant to cystoscope asymptomatic patients and it is therefore practically impossible to obtain normal tissue unless volunteers can be found to provide such tissues. As a result many of the so called normal or control specimens have come from patients with disease in the genito-urinary tract but whose bladder urothelium appeared normal by light microscopic histopathological criteria. Biopsies of normal bladder urothelium have been obtained from patients with incomplete urethral obstruction or from patients with benign prostatic hyperplasia and relatively mild urethral obstruction (Merk et al., 1977; Alroy et al., 1981).

According to one school of thought (see for instance, Melicow, 1952; Eisenberg et al., 1960; Simon et al., 1962; Cooper et al., 1973; Schade and Swinney, 1973; Skinner et al., 1974; and Knowles et al., 1980), the normal looking areas of urothelium from tumour bearing bladders are potentially neoplastic even if they exhibit normal cell differentiation.

In an attempt to locate a satisfactory source of normal bladder sample as starting material for organ culture, Knowles et al., (1980) have sampled material from patients with a wide range of clinical histories. They found that the macroscopically normal urothelium obtained from retropubic prostatectomy patients had more abnormalities than the other groups they examined, although there was no gross hyperplasia or dysplasia. This finding confirms previous observations
of Barlebo and Sorensen (1972) that 27 out of 28 prostatectomy patients they examined had abnormal or atypical bladder urothelium. A survey of the literature shows that prostatectomy patients with urine retention have provided samples of normal urothelium for several scanning electron microscope studies as well (Kjaer et al., 1976; Hodges, 1978; Nelson et al., 1979a; Jacobs et al., 1981 and others) and it would now seem necessary to treat these results with caution. Also Skoluda et al., (1972), Kumagai (1975), Price et al., (1980) and Davies and Hunt (1981) have used cytoscopic biopsy specimens of bladders with recurrent attacks of infections for scanning electron microscope studies.

To the problems created by the question of normality or otherwise of the tissue samples, must be added yet another one and this relates to the age of the patient. The age of the patient providing the tissue has seldom been mentioned in the published electron microscope accounts although it would seem that most specimens came from adult and elderly males. In the few papers where the ages were mentioned (Skoluda et al., 1972; Kjaer et al., 1976; Nelson et al., 1979a; Price et al., 1980; Davies and Hunt, 1981), there were no concerted attempts to correlate the electron microscope findings with age. This was presumably because such a correlation was not thought to be important or of any consequence. However, recent suggestions (Alroy et al., 1977; Jacob et al., 1978) that the bladder urothelium may suffer age-related changes have made it necessary to take account of this factor in electron microscope investigations of this tissue.

Considerations such as those described above make it obvious that comparison of transmission and scanning electron microscope observations of normal human bladder urothelium in the past would be both difficult and unreliable. It is also possible that some of the conflicting observations in the not too extensive literature may be due
to the diversity of samples from patients with a wide range of clinical histories. This unsatisfactory situation contrasts with the wealth of information which constitutes a mass of more firm data in laboratory mammals such as the rat, in which species, a model for chemical carcinogen-induced bladder carcinoma has also been gradually built up. The lack of similar reliable data on normal human urothelium has made it problematical to draw analogies between the rat model and naturally occurring human bladder tumours. My studies are an attempt to remedy this state of affairs and to provide a foundation for accumulating a core of baseline data on human bladder urothelium.

**OBSERVATIONS**

Group I. **Normal Bladder Urothelium of Young Adults Ranging in Age from 21 to 31 Years**

In light microscope preparations, the urothelium could be seen to contain 5 layers of cells and rarely 6. The superficial layer consisted of large cells with oval nuclei and each of them overlay two, three or more cells of the intermediate layers. The cells of the intermediate and basal layers were smaller and more spherical. The lamina propria immediately beneath the basal lamina contained scattered fibroblasts and some blood capillaries. The deeper tissue contained large blood vessels embedded in the connective tissue (Fig. 34).

**TEM observations - General** In electron micrographs, the large superficial cells were generally of low electron density and were mono- (Fig. 35) or bi-nucleated (Fig. 36). They showed large clusters of mitochondria in their apical cytoplasm close to the luminal surface. The
nuclei had smooth outline and contained chromatin in a highly dispersed state amidst which several nuclear inclusions were often visible. The nucleoli were characterised by distinct nucleolonemas (Fig. 36). The lateral borders of the superficial cells were closely apposed to each other with hardly any intercellular space (Fig. 35); desmosomes were noticeable periodically along the border. At the luminal surface, adjoining superficial cells were held together by the so called terminal bar which consisted of a multiple complex of cell junctions - the zonula occludens or tight junction, zonula adherens or intermediate junction and macula adherens or desmosome (Fig. 37). In suitable sections passing through the entire junctional complex, the tight junction, formed by the fusion of the outer leaflets of the adjacent plasma membranes, measured about 18 nm in width and up to 400 nm in length. Immediately below, the lateral surfaces were held together by zonula adherens, also about 400 nm long, where the two plasma membranes were aligned in parallel but separated from each other by an interspace measuring about 25 nm in width. The interspace seemed to be filled with a fine electron dense filamentous material. In the subjacent cytoplasm, a mat of thicker filaments was present. The desmosome, situated deeper in the cell, consisted of two parallel membranes separated by an interspace of about 30 nm in width, and hence wider than that of the zonula adherens. Apart from the dense fibrillar plaques in the subjacent cytoplasm, a central electron dense stratum was present in the intercellular space.

The three or more layers of intermediate cells were of uniform appearance and size (Fig. 39). They were more electron dense than the superficial cells (Fig. 38). The nuclei of these cells were large, relative to the size of the cell, and had a more irregular outline
than those of the superficial cells. The structure and distribution of chromatin were different from that of the superficial cell in that a portion of the chromatin was highly dispersed and confined to the centre of the nucleus while the somewhat less dispersed chromatin was distributed along the nuclear periphery. The nucleolonema of the nucleoli in the intermediate cells was generally not so distinct as in the superficial cells (Fig. 39). In low magnification micrographs, mitochondria stood out as the main organelle in the cytoplasm (Fig. 39) and they appeared to be preferentially localised in the apical part of the cells, particularly in those cells adjoining the superficial cell. The apical cytoplasm of these cells also exhibited a number of small vesicles which were situated close to the plasma membrane facing the superficial cell. The apical border of the topmost layer of intermediate cells was closely apposed to that of the superficial cell without any intervening space, but the lateral and basal borders showed distinct intercellular spaces in which many long interdigitating cell surface processes were visible. In places, these processes were joined by desmosomes.

The basal cells were the most electron dense cells of the urothelium and they tended to be arranged perpendicularly to the basal lamina (Fig. 40). Their nuclei were even more irregular in shape and the invaginations of the nuclear envelope were often so deep as to give the cells a multilobate appearance. The chromatin of these cells was in a more condensed state than in cells of the overlying layers and it was more or less uniformly distributed throughout the nucleus. The nucleoli of these cells were small bodies with indistinct nucleolonema (Fig. 40). In low power pictures, mitochondria were the main organelles seen in the cytoplasm and they were scattered
throughout the cell. The space between the cells was wider than that between the intermediate cells and the thin microvilli-like cell surface processes were longer (Fig. 40). A general impression gained from examination of a large number of electron micrographs of basal cells was that desmosomes between the interdigitating plasma membrane processes of adjoining cells occurred less frequently than in the case of intermediate cells. The basal cells rested on the basal lamina to which they were attached at various intervals by hemi-desmosomes. As described in other epithelial-stromal interfaces, hemi-desmosomes were patchy accumulations of electron dense material like half of a desmosome. At the basal lamina level of the urothelium there were no tight junctions between the adjoining cells to isolate the intercellular space, or the extracellular compartment of the urothelium, from the underlying lamina propria.

The basal lamina was closely applied to and followed the contours of the plasma membrane of the basal cell. In the transmission electron microscope, the structure resolved into a lamina lucida and a lamina densa (Fig. 40). The lamina lucida measured about 30 nm in thickness but narrowed to about 22 nm in the regions of the hemi-desmosomes. The lamina densa was much thicker, measuring about 0.2 μm and appeared to be made up of a network of diffusely flocculent material (Figs. 40 and 41).

SEM Observations on the Luminal Surface. In low power scanning electron micrographs, the superficial cells of the moderately stretched urothelium appeared as uniformly smooth-surfaced cells (Fig. 46) with the boundaries between cells appearing as slightly elevated ridges.
At higher magnification, it could be seen that the cells were polygonal in shape and also that the surface was characterised by a simple pattern of microridges (Fig. 47). That these microridges were fairly shallow was further illustrated in high magnification such as that shown in Fig. 48. Also visible occasionally in such scanning micrographs were globular projections amidst the microridges.

TEM Observations. The luminal surface and the superficial cells. A representative micrograph of the apical portion of a superficial cell and the luminal plasma membrane is presented in Fig. 42. Several vesicles were present in the region of cytoplasm immediately below the luminal membrane. They were either spherical or elongated, and there were hardly any which could be described as typically fusiform. The elongated vesicle in Fig. 42 is nearly fusiform in shape. Another similar instance is shown in Fig. 49.

The luminal membrane in many places resolved into the classical unit membrane structure containing two electron-dense leaflets enclosing a central electron-lucent layer. In most instances, the unit membrane structure was asymmetrical with one of the leaflets - the outer one facing the lumen - being slightly thicker than the other facing the cytoplasm; the total thickness of the membrane was about 12 nm (Figs. 43, 44). In other instances, the membrane appeared symmetrical with both the leaflets of equal thickness and a total thickness of approximately 10 nm (Fig. 45). Since one could not expect the tissue to be cut in a plane perfectly perpendicular to the urothelium all the time, it was not possible to make any precise estimate of the relative extents of the asymmetrical and symmetrical stretches of the membrane. However, the impression gained by examining a large number of micrographs
was that the major part of the luminal membrane was asymmetrical. The cytoplasmic vesicles described above showed either symmetrical or asymmetrical limiting membrane (Figs. 42, 49).

The luminal surface generally had a smooth contour and the instances nearest to a scalloped appearance that could be found are shown in Figs. 43, 44; in both these instances, it could also be noted that the luminal membrane had an asymmetrical unit membrane structure. The outer surface of the luminal plasma membrane, whether symmetrical or asymmetrical in structure, was covered by a finely filamentous glyco-calyx, as shown in ruthenium red-stained preparations (Figs. 43, 44 and 45).

The Golgi complexes in the superficial cells were extensive and well developed with stacks of dilated cisternal elements, free vesicles of various dimensions, and also coated vesicles. Some of the coated vesicles were free and others appeared to be fusing with or budding from large Golgi cisternae (Fig. 50). The cells invariably contained several large polymorphic bodies with extremely heterogeneous content of varying electron density, and also some smaller uniformly less dense bodies or vesicles with a homogeneous content. Scattered microfilaments as seen in Fig. 50 were also present. The microfilament system was probably part of the cytoskeletal system linked to the cellular junctions, mainly the desmosomes. The endoplasmic reticulum appeared to be meagre in the superficial cells and it was represented by randomly oriented cisternae. The cytoplasmic matrix contained large numbers of free ribosomes. As stated earlier, the mitochondria were mainly concentrated in the apical portions of the cells. The majority of them had rounded profiles and measured 0.3 μm in average diameter; a few elongated forms did occur and they measured
up to about 0.5 \mu m in length. The cristae were usually arranged transversely although a longitudinal arrangement was sometimes noticeable. The mitochondrial matrix was more electron dense than the cytoplasmic matrix.

**Intermediate cells.** The apical cytoplasm of the top layers of intermediate cells had certain features in common with the superficial cells described above. These were the mitochondrial clusters and the large numbers of vesicles (Fig. 39). Most of the vesicles were spherical and their limiting membrane had a symmetrical structure. A few vesicles, some of which were oval shaped, were seen in high power micrographs to have an asymmetrical limiting membrane. The plasma membrane facing the overlying superficial cell displayed long, thin extensions resembling microvilli which were so closely apposed with similar processes of the superficial cell, that no intercellular space was noticeable. On the lateral borders of adjoining intermediate cells, similar cell surface processes were present and although some of them interdigitated, others could be seen protruding freely into a wide intercellular space (Figs. 38, 39). Along all borders desmosomes were observable.

The intermediate cells, especially those lying adjacent to the superficial cells had another feature in common with the superficial cells; this was not just the presence of large polymorphic bodies, but the presence in large numbers of such bodies. Smaller bodies of homogeneous consistency were also noticeable in the cell. The concentration of mitochondria in the apical cytoplasm was seen even in some of the lower layers of intermediate cells but the spherical vesicles mentioned above were not. Instead, the vesicles were
noticeable more in the lateral portion of the cell, often close to the Golgi apparatus. The Golgi apparatus in intermediate cells generally were well developed (Fig. 51) and displayed several components including large vesicles and small coated vesicles, as in the superficial cells. The membranes of the Golgi components and the free vesicles in their proximity appeared to be symmetrical in structure. Polymorphic bodies were observable in all layers of intermediate cells, but my impression was that more of them per cell occurred in the top layers of intermediate cells, especially in that adjacent to the superficial cells. Rough endoplasmic reticulum in all the intermediate cells was represented by scattered cisternal profiles, as in the superficial cells. The cytoplasmic matrix contained many ribosomes and microfilaments as described in the superficial cells. Meandering microtubules were also discernible in several micrographs.

**Basal cells.** The small size of these cells, their electron density and highly condensed chromatin marked them out as a cell type different from the overlying intermediate cells. The deep indentations of the nuclear envelope were also a striking feature which was not shared by other cells of the urothelium. The cell borders displayed a number of long microvilli which loosely intertwined with their counterparts from adjoining cells (Fig. 40). The interspaces between the basal cells constituted the widest part of the extracellular compartment of the urothelium. In addition, it seemed that the microvilli of these cells were much longer than those of the intermediate cells; some of the microvilli measured over 2 μm.

The mitochondria of the basal cells were distributed throughout the cytoplasm and were the conspicuous organelles in the cell. The
Golgi apparatus was small and consisted of few cisternae and free vesicles which did not include the coated variety. Widely separated profiles of cisternae of rough endoplasmic reticulum could be made out amidst the numerous free ribosomes. Typical polymorphic bodies with highly heterogeneous content, as in superficial and intermediate cells, were not encountered in the basal cells. Instead, the primary lysosome-like bodies present were small and their contents were very homogeneous and of uniformly low electron density.

**Morphometric analysis.** The data relating to the mean profile areas of the three types of urothelial cells and their nuclei are presented in Tables 9 and 10 and text Figs. 9 and 10. The superficial cell is approximately 6 times larger than the basal cell and about 3 times larger than the intermediate cells. The difference in the size of the nucleus of the superficial cell is less dramatic; it is nearly 3 times larger than the nucleus of the basal cell and about 2 times larger than that of the intermediate cell. As a result of these differences in the relative sizes of the cells and their nuclei, the nucleo-cytoplasmic ratios vary from 1:5 in the superficial cell to 1:2.5 in the intermediate cell and 1:1 in the basal cell (Table 11 and text Figure 11). The relatively large size of the cytoplasm of the superficial cell is a noteworthy feature.

The mean volume density values of the Golgi complex and the various plasma membrane-related vesicles derived from the Golgi in the three cell types are presented in Table 12 and text Fig. 12. The highest value for this family of membraneous structures is found in the superficial cells (in spite of the larger volume of the cytoplasm of these cells) compared with the intermediate and basal cells.
The volume density data for mitochondria and endoplasmic reticulum are shown in Tables and text Figs. 13 and 14 respectively; the values for these organelles fluctuate within narrow limits between the different urothelial cells. Data for the small primary lysosome-like bodies (mainly in the basal cells) and the larger polymorphic bodies (mainly in the intermediate and superficial cells) show (Table 15 and text Fig. 15) that the volume fractions occupied by these bodies are the lowest in the basal cells. The value is higher in the intermediate cells than in the superficial cells and this is surprising. Table 16 and text Fig. 16 show that the volume density of the cytoplasmic matrix is, as expected, the highest in the undifferentiated basal cell compared with the other two cell types. It is noteworthy, however, that the volume densities of the matrix are not very different in the intermediate and superficial cells of this urothelium.

Group II. Normal Bladder Urothelium of Middle-Aged Subjects Ranging in Age from 35 to 49 Years

Light microscope preparations cut in the most suitable plane, i.e., at right angles to the bladder wall, showed the urothelium to be 4 to 5 layers thick (Fig. 52). The superficial cells were large with oval nuclei as in the previous age group and each one of them straddled two or more of the underlying intermediate cells. Both in light microscope preparations and in low power electron micrographs, the general appearance of the urothelium and the dispositions of the cells (Fig. 53) were as in the urothelium of the previous age group.
TEM observation - General. The superficial cells were of lower electron density than the underlying cells and most of them appeared to be mononucleated (Fig. 55). Binucleated cells were seen but they were rare (Fig. 54). Clouds of mitochondria were frequently visible in the apical cytoplasm (Fig. 55) between the nucleus and the luminal membrane. The smooth nuclear outline, the finely dispersed chromatin and prominent nucleolonema were other features the superficial cells shared with their counterparts in the urothelium of the previous age group described. Electron micrographs such as that in Fig. 55 clearly showed that there was practically no interspace between adjoining superficial cells, and that the apposed cell membranes were held together at the luminal surface by tight junctions (Figs. 59, 60). As described earlier, the tight junction was followed by a zonula adherens and a macula adherens to form a complex of attachment devices. As stated earlier, such a complex of junctions has been referred to in the early literature dealing with epithelial cells as a terminal bar. In instances where an intermediate cell could be seen jutting to the lumen between superficial cells (Fig. 61), the junctional complex appeared to be absent and the barrier between the lumen and the intercellular space was formed by adherens junctions only (Fig. 62).

The intermediate and basal cells appeared in low power micrographs as more or less similar in electron density (Fig. 53). The nuclear envelope of the intermediate cells was more irregular than that of superficial cells and the irregularity was much more pronounced in the basal cells which at times seemed to be multilobate because of deep invaginations of the envelope. The nature and distribution of chromatin and the appearance of nucleoli in the intermediate and
basal cells were as described before in the urothelium of young adults.

The mitochondria of the intermediate cells were clustered in the apical cytoplasm (Fig. 55) - a feature also noted in the urothelium of the younger subjects. Unlike in the younger tissue, however, the apical cytoplasm of the intermediate cells, of even those adjoining the superficial cells did not contain many vesicles. Only in the occasional instances where an intermediate cell protruded into the lumen between superficial cells could one notice the vesicles in the apical cytoplasm (Figs. 61, 62).

An intercellular space between the superficial cells was almost non-existent but it was generally pronounced between the intermediate cells and even more so in the deeper region of the urothelium (Figs. 57, 58), where long, slender microvilli could be seen projecting into it. Desmosomes were noticeable along the various cell borders of intermediate cells at an average frequency of about one per 1.0 to 1.5 μm. Fewer of these junctions occurred between basal cells and between basal and intermediate cells.

The basal cells, the basal lamina and the underlying connective tissue appeared as in the urothelium of the previous age group. At the basal lamina level, the intercellular space between basal cells appeared to be open, there being no sealing junctions (Figs. 58, 64).

SEM observation on the luminal surface. In low power scanning micrographs, the luminal surface presented a variable appearance. In places, the surface appeared smooth and apparently free of microridges (Fig. 67). In other areas, the surface displayed a simple pattern of microridges: the ridges were short and discontinuous as shown in Fig. 69. Also noticeable in such areas were short, scattered microvilli amidst the ridges. A good example of the
variability and the range of surface features observable in the urothelium of this age group is presented in Fig. 68.

TEM observations. The luminal surface and the superficial cells. In thin sections, as in the SEM preparations, the appearance of the luminal surface was variable. Part of the surface with a smooth outline is shown in Fig. 56. This micrograph quite clearly illustrates that the luminal membrane is asymmetric for the most part, i.e., that its outer leaflet facing the lumen is thicker than the inner leaflet facing the cell cytoplasm. However, in other instances where the surface displayed scattered microvilli the luminal membrane could be seen to be symmetric (Fig. 66). In areas where the surface was thrown into shallow troughs, there was a semblance of a scalloped contour (Fig. 54), but in such areas also, the luminal membrane was, in part, asymmetric (Fig. 65) and in part symmetric (Fig. 70).

In preparations stained with ruthenium red, the outer leaflet of the luminal plasma membrane was covered with a fine fibrillar glyco-calyx interspersed with beads of material at irregular intervals (Figs. 56, 65); the glycocalyx was observable over the asymmetrical and symmetrical stretches of the membrane (Figs. 56, 65, 72, 73).

The apical cytoplasm of the superficial cells contained large numbers of mitochondria as mentioned earlier, but what was striking was the prolific occurrence of vesicles of various sizes and shapes (Figs. 65, 66, 70, 71). They were mainly rounded or oblong with a sprinkling of elongated and oval forms (nearly fusiform) scattered at random. It is probably of some significance that in spite of the abundance of free vesicles in the cytoplasm, typical fusiform vesicles as in the rat, were relatively rare. The limiting membrane of the more
elongated vesicles tended to be asymmetric with the thicker leaflet lining the lumen (Figs. 70, 71). It is interesting to note that the rounded or oblong vesicles also tended to have asymmetrical limiting membrane, although a symmetrical limiting membrane was also observable in some instances.

The Golgi complex was very extensive and prominent in the superficial cells (Fig. 74). In the immediate vicinity of the complex, profiles of rough endoplasmic reticulum were often present. The Golgi complex consisted of large flattened saccules and vesicles of various sizes. Also noticeable were fairly large numbers of coated vesicles, some free and others in contact with or continuous with smooth membraned vesicles. In areas where the rough endoplasmic reticulum and Golgi apparatus were closely intermixed, it was difficult to assign some of the smooth or partially smooth vesicular elements to one or the other of the membrane systems. In the section shown (Fig. 74) there are visible especially in peripheral regions of the Golgi apparatus several large vesicles, including a nearly fusiform vesicle. The structure of the limiting membrane of these vesicles is not clear in this preparation, but in other high resolution micrographs of superficial cells where similar free vesicles of various shapes were present in the Golgi zone, it was obvious that they were the same kind of vesicles with asymmetric or symmetric limiting membrane as those found so abundantly in the apical cytoplasm. In the figure shown (Fig. 74) there was many coated vesicles.

The organisation and distribution of other organelles such as the rough endoplasmic reticulum and mitochondria were as in the superficial cells of the urothelium of the younger age group.
Intermediate cells. The 2 to 3 layers of intermediate cells present in the urothelium of the age group under consideration were remarkably similar in their ultrastructural features. The clustering of mitochondria in the apical regions of the cytoplasm, evident even in low power micrographs, was mentioned earlier. It was also pointed out, how occasionally an intermediate cell could be observed jutting into the lumen between superficial cells (Fig. 61). In such instances, the cell exhibited a feature that was common in superficial cells but not otherwise evident in intermediate cells situated in lower layers. The feature in question was the presence of free vesicles, either spherical or elongated in shape. Low power micrographs showed that the distribution of the vesicles was not confined to the cytoplasm immediately adjacent to the luminal membrane but extended to the rest of the apical cytoplasm.

Although free vesicles of the type described above were not usually evident in the intermediate cells, the Golgi apparatuses were moderately developed (Fig. 75). The Golgi system consisted predominantly of flat saccules and few free vesicles. Some coated vesicles were also noted in the area.

Polymorphic bodies, some of them resembling lipofuscin granules, scattered profiles of rough endoplasmic reticulum, many monoribosomes, microfilaments and microtubules were the other contents of the intermediate cells.

Basal cells. The basal cells of this urothelium did not appear to be any different from their counterparts in the urothelium of the younger urothelium. They were the smallest of the urothelial cells, and their highly irregular nuclei contained chromatin that was more
condensed than the other two cell types (Fig. 58). Their nucleo-cytoplasmic ratio was the highest of all urothelial cells and their organelle content was the poorest. The cytoplasm was dominated by free ribosomes - predominantly monoribosomes. Profiles of rough endoplasmic reticulum were scarce and the Golgi apparatus was small and localised in the basal region of the cell. Mitochondria were scattered throughout the cell. Occasionally, bodies reminiscent of primary lysosomes could be made out.

**Morphometric analysis.** The mean profile cell areas of the superficial, intermediate and basal cell types of the urothelium of middle-aged subjects are shown in Table 9 and text Fig. 9. The cells are not significantly different in size from those of the younger urothelium. The nuclei of the superficial, intermediate and basal cells show practically no difference in size when compared with their counterparts in the urothelium of the younger age group (Table 10 and text Fig. 10). As a result, there is no difference in the nucleo-cytoplasmic ratios of the three cell types when compared with those of the previous age group (Table 11 and text Fig. 11).

The volume density of the Golgi complex and the related free vesicles in the apical cytoplasm of the superficial cell is the highest of all urothelial cells in this group, and the least value occurs in the basal cells (Table 12 and text Fig. 12). The moderately well developed nature of the Golgi complex described earlier in the intermediate cells is reflected in the volume density value which is almost three times greater than that in the basal cell and nearly half of that of the superficial cell.

The volume fractions occupied by the group of bodies referred to
as polymorphic bodies (mainly in the intermediate and superficial cells) and primary lysosomes (mainly in the basal cell) are shown in text Fig. 15 and Table 15. More or less similar volume fractions of the superficial and intermediate cells are occupied by this group of membrane-limited bodies, but, as expected from initial study of random micrographs, the fraction is very low in the basal cells.

The volume densities of rough E.R. are similar in the superficial and intermediate cells but lower in the basal cells (Table 14 and text Fig. 14), while the density of mitochondria does not differ markedly between the three cell types (Table 13 and text Fig. 13).

Group III. Normal Bladder Urothelium of the Elderly Ranging in Age from 58 to 82 Years

Examination of sections cut in different planes for light microscopy revealed that there were hardly any regions in the urothelium which were more than 3 or 4 cell layers thick (Fig. 76a). In several instances, only one or two layers were present (Fig. 76b) interspersed between 3-4 cells thick regions. In places where the urothelium was 3-4 cells thick, the superficial cells were smaller than those of previous age groups.

TEM observations - General. An electron micrograph of a superficial cell in an area of the urothelium containing 3-4 cell layers is shown in Fig. 77. The cell as a whole was of low electron density and, as in previous age groups, the nuclei had a smooth outline and the chromatin was in a highly dispersed state. This cell also showed a number of round vesicles in the apical cytoplasm immediately adjacent
to the luminal membrane. This superficial cell adjoined a thin stretch of urothelium (towards the right of Fig. 77) consisting of two smaller cells. The cell facing the lumen at the thin stretches sometimes resembled the intermediate cell of the thick regions. The cell borders between all these cells nevertheless appeared close and normal, there being practically no interspace between them. Large clusters of mitochondria were prominent in the apical cytoplasm of the two smaller cells. Another noteworthy feature of the urothelium of this age group was the widespread occurrence of cells of differing electron density often adjoining one another (Figs. 79, 80). The difference in density was not merely due to the presence of variations in the quantity of glycogen, but the dark and light cells differed in the inherent density of their cytoplasm.

Deeper regions of the thick regions showed variable features. In some instances, the intermediate and basal cells were present (Fig. 78, left half of the micrograph) as in the urothelia of younger subjects, but in other instances (Fig. 78, right half of the picture), the urothelium appeared to include infiltrating lymphocytes or other elements of the connective tissue.

The variations in thickness of the urothelium in this group of specimens is further illustrated in Figs. 79 and 80. On occasions, the basal lamina appeared bare or devoid of urothelium. The cells facing the lumen of the bladder in 2 cell thick regions were of the same size and ultrastructural morphology as those at the base of the urothelium in thicker regions. In many instances, just one layer of cells were seen spanning the entire width of the urothelium. One feature common to all these cells and observable in low power electron micrographs was the abundance of mitochondria in
the apical cytoplasm of such cells (Figs. 79 and 80). The intermediate and basal cells in thick regions (part of Fig. 78) were as described previously in the urothelia of the adult and middle aged subjects. The basal lamina and the underlying connective tissue also seemed to be as in previous age groups; a thick lamina densa measuring over 0.2 μm was present in some preparations, as was also observed in young adults.

In several specimens of this age group the urothelial cells contained varying amounts of glycogen.

**SEM observations on the luminal surface.** In moderately stretched, scanning microscope preparations, the cells appeared slightly variable in size and the pavement-like pattern of the polygonal cells was not so prominent as in younger subjects (Figs. 86, 87). In several locations small areas of underlying cells could be noticed jutting between the surface layer of cells, and this feature was most pronounced in this group of urothelia than in any other already described. In low power scanning micrographs the cells generally displayed a pattern of fine microridges between the elevated cell borders (Fig. 87), but higher power pictures (Fig. 88) often showed the presence of sparsely distributed microvilli of uniformly small size.

**TEM observations.** The luminal surface and the superficial cells. The appearance of the luminal surface as a whole was more uniform in this age group than in any other urothelia examined in this study, and many preparations showed the presence of microvilli. In the thin urothelial regions, the surface was microvillous (Figs. 79, 80), and high resolution micrographs (Fig. 81) clearly demonstrated that the luminal
plasma membrane had a symmetrical structure with an overall thickness of about 9-10 nm. A typical example of the junctions met with in the thin urothelial regions is shown in Fig. 82. Although the adjoining cells forming such regions were closely apposed and there was hardly any intercellular space (Figs. 79, 80), the cells did not appear to be held together at the luminal surface by the usual zonula occludens type of junction. The site in question often displayed one or several closely placed adherens type of junctions with subjacent cytoplasmic densities as shown in Fig. 82.

The cells that formed the thin regions showed no special features of cytodifferentiation (Figs. 79, 80). As stated earlier, they showed large numbers of mitochondria in their apical regions, but apart from these, no other organelles were prominent. The small Golgi apparatus was situated lateral to the nucleus but more towards the basal region of the cell (Fig. 79). The cells in the unusually thin stretches of the urothelium also tended to be columnar in shape and their nuclei showed masses of condensed chromatin especially along the nuclear margin.

In the thick regions (3-4 cells) of the urothelium, a more or less normal pattern of cytodifferentiation was evident. But the luminal surface presented varying contours. The one shown in Fig. 83 clearly indicated blunt microvilli; the microvilli were rather widely spaced and their limiting membrane which was about 9-10 nm thick displayed a symmetrical structure. Another surface contour observed was as in Figs. 84 and 85, which gave the impression of a scalloped surface; however in such instances also the luminal membrane showed only a symmetrical structure and the crests were drawn into microvilli.
Binucleated superficial cells were not observed in this group. Free vesicles were not numerous in the apical cytoplasm of the superficial cells of the thick urothelium (Fig. 77). On occasions where such vesicles were present, they were either round or laterally compressed entities (Fig. 77, 85, 89) and in both types the limiting membranes were symmetrical in structure. Typical fusiform vesicles were very rarely met with in specimens of this age group. The superficial cells contained polymorphic bodies and sometimes lipofuscin granules as described in the previous age group.

The intercellular junctions between the superficial cells at their luminal surface were carefully examined in the thick regions of the urothelium and the impression gained was that a typical zonula occludens is not always present. In many instances, the appearance of the junction (Fig. 83) closely resembled a zonula occludens (tight junction) but close examination suggested that there might be incomplete fusion of the outer leaflets of the adjoining membranes. However, in other instances typical tight junctions were observed and they resembled the tight junctions in the urothelia of younger age groups. Below the zonula occludens or occludens-type junction, there were present adherens junctions with dense cytoplasmic plaques as in a normal junctional complex.

The ruthenium red-treated preparations showed a thick surface coat mainly in the form of dense beads of material over the luminal plasma membrane (Fig. 89) of the thick region of the urothelium. The surface coat was more prominent than in the previous age group. It was not only thicker but also more beaded in structure than that observed in the urothelium of the middle aged.

Well developed Golgi complexes were noted in the superficial
cells of the thick urothelial regions. An area of Golgi complex is shown in Fig. 90. Apart from the closely spaced cisternae, a prominent feature of the Golgi complex was the presence of many vesicles of various sizes and electron density, including coated vesicles. The larger, uniformly dense vesicles present in the section (Fig. 90) are probably primary lysosomes. Profiles of rough endoplasmic reticulum were often noticed in the vicinity of Golgi complexes.

**Intermediate cells.** A typical intermediate cell like that observable in young adults and in middle aged subjects was less likely to be found in the aged urothelium since in many areas the urothelium was thin with only one or two cells. Even in the thick areas, the intermediate cells (Figs. 77, 78) did not appear to be as well differentiated as in the urothelia of younger age groups, although certain features such as clusters of mitochondria were noticeable in the apical cytoplasm. In some, but not all, of the intermediate cells, the Golgi areas presented a picture somewhat similar to that described above in the superficial cells; the extent of the Golgi area was, however, more restricted. Polymorphic bodies were also occasionally visible in the intermediate cells.

**Basal cells.** The basal cells of the urothelium of this age group (Fig. 78) did not appear to be any different from their counterparts in the urothelia described previously. But, at times, what was apparently an undifferentiated basal cell, constituted the entire thickness of the urothelium (Figs. 79, 80). In such instances, the cell was vertically stretched to become columnar and the nuclei also
took up a vertical orientation. The Golgi apparatus in such cells did not appear to be any better developed than in typical basal cells of 3-layered urothelial regions, but it was often found to be displaced to a lateral location from its usual basal location. Another difference was that there seemed to be many more mitochondria in these cells than usual and clusters of them were found in the apical cytoplasm instead of being distributed throughout the cytoplasm.

A phenomenon which was observed in this age group with greater frequency than in the other groups was pinocytotic activity especially along the lateral borders of urothelial cells (Fig. 91). A small pit-like depression along the plasma membrane probably marked the beginning of the process (Fig. 91a). The invagination gradually seemed to deepen (Fig. 91b) resulting finally in the formation of a free vesicle up to about 80 nm in diameter (Fig. 91c). Examination of high power pictures suggested that the pits and vesicles were similar to what have been called coated pits and coated vesicles in the literature. At first, the newly formed vesicles seemed to possess a moderately dense and fuzzy content, somewhat like that in the intercellular space, but subsequently the content appeared denser and filled the vesicle fully or partially (Fig. 91d). The presence of varying numbers of coated vesicles in the Golgi complex regions of superficial and intermediate cells was mentioned earlier. Coated vesicles were also found in the urothelial cells of the younger urothelia examined in this study.

**Morphometric analysis.** The mean profile area of the superficial cell in the thick urothelial regions of this age group is significantly lower than those of the middle age and young adult groups. The intermediate cells, where such occur, are more or less of similar size as in the younger urothelia and the basal cells in the thick urothelial
Table (9).  Cell area of the normal human urothelium in $\mu m^2$.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young adult</th>
<th>Middle-aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>653.000 ± 32.300</td>
<td>568.750 ± 28.826</td>
<td>396.250 ± 30.937</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>P&lt;0.01</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Intermediate</td>
<td>222.500 ± 15.703</td>
<td>207.500 ± 16.780</td>
<td>188.750 ± 19.242</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Basal</td>
<td>96.250 ± 7.557</td>
<td>90.000 ± 8.732</td>
<td>87.500 ± 8.750</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Table (10). Nuclear area of normal human urothelium in $\mu m^2$

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young adult</th>
<th>Middle-aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>110.000 ± 6.928</td>
<td>105.000 ± 7.624</td>
<td>103.750 ± 10.400</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Intermediate</td>
<td>63.750 ± 5.292</td>
<td>61.250 ± 4.136</td>
<td>58.750 ± 6.441</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Basal</td>
<td>47.500 ± 4.294</td>
<td>48.750 ± 5.722</td>
<td>47.500 ± 4.969</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Table (11)  Nuclear cytoplasmic ratio of normal human urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young adult</th>
<th>Middle-aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.204 ± 0.014</td>
<td>0.226 ± 0.005</td>
<td>0.355 ± 0.038</td>
</tr>
<tr>
<td></td>
<td>(1:4.902)</td>
<td>(1:4.425)</td>
<td>(1:2.817)</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>P&lt;0.010</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.402 ± 0.041</td>
<td>0.419 ± 0.048</td>
<td>0.452 ± 0.066</td>
</tr>
<tr>
<td></td>
<td>(1:2.488)</td>
<td>(1:2.387)</td>
<td>(1:2.212)</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Basal</td>
<td>0.974 ± 0.186</td>
<td>1.182 ± 0.122</td>
<td>1.188 ± 0.133</td>
</tr>
<tr>
<td></td>
<td>(1:1.027)</td>
<td>(1:0.846)</td>
<td>(1:0.842)</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
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</table>

Table (12)  Volume density of Golgi body and vesicles in normal human urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young adult</th>
<th>Middle aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.096 ± 0.010</td>
<td>0.063 ± 0.008</td>
<td>0.091 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>P&lt;0.001</td>
<td>P&lt;0.001</td>
<td>N.S.</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.37 ± 0.010</td>
<td>0.029 ± 0.007</td>
<td>0.020 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>P&lt;0.005</td>
</tr>
<tr>
<td>Basal</td>
<td>0.014 ± 0.007</td>
<td>0.011 ± 0.005</td>
<td>0.009 ± 0.000</td>
</tr>
<tr>
<td></td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Table (13) Volume density of mitochondria in normal human urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young adult</th>
<th>Middle-aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.115 ± 0.009</td>
<td>0.155 ± 0.020</td>
<td>0.093 ± 0.011</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.142 ± 0.019</td>
<td>0.155 ± 0.015</td>
<td>0.073 ± 0.013</td>
</tr>
<tr>
<td>Basal</td>
<td>0.096 ± 0.012</td>
<td>0.117 ± 0.017</td>
<td>0.054 ± 0.007</td>
</tr>
</tbody>
</table>

Table (14) Volume density of endoplasmic reticulum in normal human urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young adult</th>
<th>Middle-aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.012 ± 0.002</td>
<td>0.036 ± 0.006</td>
<td>0.014 ± 0.005</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.017 ± 0.003</td>
<td>0.048 ± 0.005</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>Basal</td>
<td>0.014 ± 0.000</td>
<td>0.019 ± 0.006</td>
<td>0.011 ± 0.000</td>
</tr>
</tbody>
</table>
Table (15) Volume density of polymorphic bodies in normal human urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young adult</th>
<th>Middle-aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.034 ± 0.013</td>
<td>0.036 ± 0.005</td>
<td>0.041 ± 0.012</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.053 ± 0.011</td>
<td>0.037 ± 0.007</td>
<td>0.048 ± 0.015</td>
</tr>
<tr>
<td>Basal</td>
<td>0.007 ± 0.001</td>
<td>0.012 ± 0.005</td>
<td>0.010 ± 0.000</td>
</tr>
</tbody>
</table>

Table (16) Volume density of cytoplasmic matrix in normal human urothelium.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Young adult</th>
<th>Middle-aged</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>0.393 ± 0.030</td>
<td>0.476 ± 0.031</td>
<td>0.496 ± 0.015</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.458 ± 0.058</td>
<td>0.526 ± 0.034</td>
<td>0.566 ± 0.024</td>
</tr>
<tr>
<td>Basal</td>
<td>0.527 ± 0.022</td>
<td>0.647 ± 0.029</td>
<td>0.736 ± 0.026</td>
</tr>
</tbody>
</table>
FIG. (9) THE CELL AREA OF THE NORMAL HUMAN UROTHELIUM.

FIG. (10) THE NUCLEAR AREA OF THE NORMAL HUMAN UROTHELIUM.

FIG. (11) THE NUCLEAR CYTOPLASMIC RATIO OF THE NORMAL HUMAN UROTHELIUM.

FIG. (12) THE VOLUME DENSITY OF GOLGI BODIES AND VESICLES IN NORMAL HUMAN UROTHELIUM.
Fig. (i3) The volume density of mitochondria of the normal human urothelium.

Fig. (i4) The volume density of e.r. of the normal human urothelium.

Fig. (i5) The volume density of polymorphic bodies of the normal human urothelium.

Fig. (i6) The volume density of cytoplasmic matrix of the normal human urothelium.
regions are practically of the same size as in the younger urothelia (Table 9 and text Fig. 9).

The mean area of the nucleus of the superficial cells is not significantly different from that of the middle aged and young adult. The data (Table 10 and text Fig. 10) show that the nuclei of intermediate and basal cells are practically of the same size as in the younger urothelia studied. As a result of the smaller cytoplasmic area of the superficial cells of this age group, the nucleo-cytoplasmic ratio of these cells rises to 1:3 from the ratio of 1:5 in the previous groups. The ratios of the intermediate and basal cells in the thick region of the urothelium are practically unchanged (Table 11 and text Fig. 11).

Data pertaining to the volume density of various other organelles in the three cell types are presented in Tables 12-16 and text Figs. 12-16. The volume density of the Golgi complex and the various associated vesicles in the superficial cell is comparable to that in the young adult (Table 12 and text Fig. 12) while that of the polymorphic bodies including lipofuscin granules and the primary lysosome-like bodies (Table 15 and text Fig. 15) is comparable to those in the urothelia of the younger age groups.

DISCUSSION

This is the first attempt to study the ultrastructural morphology of human bladder urothelium from different age groups in order to find out if any variations occur with age. In the past, the relatively few studies on the ultrastructure of human urothelium were primarily for purposes of comparison with the urothelia of rodents and other mammals.
The earliest investigators Kashiwai et al., 1963; Battifora et al., 1964; Monis and Zambrano, 1968) gave little information on the source of the tissue and practically none on the age of the donor. In most of the subsequent publications as well, information on the age of the patients from whom the specimens were obtained was seldom provided. The present study of biopsies from three different age groups covering most of the human life span shows that there are structural differences in the urothelium of the different age groups.

The maximum number of cell layers present in the urothelia of all age groups that I have examined is five or perhaps six, and this condition prevails in the young adult, that is, the youngest age group examined in the present study. The urothelia of the oldest group tends, as a whole, to be thinner with no regions exhibiting more than three to four layers. Many stretches contain fewer than three layers of cells and in such areas the urothelium may contain only one or two layers. Occasionally, the basal lamina has even appeared bare. The urothelium of the middle aged group consisting of four to five cell layers may then be regarded as intermediate in thickness between those of the younger and the older age groups. A survey of the literature reveals that there is no general agreement on the thickness of the normal human bladder urothelium. Some of the previous workers (Kashiwai et al., 1963; Battifora et al., 1964; Monis and Zambrano, 1968; Fulker et al., 1971; Firth and Hicks, 1973; Tannenbaum et al., 1978; Tannenbaum, 1979) imply or state that it is three or four cells thick while according to others, such as Newman and Hicks (1977), the tissue is four to five cells thick. Koss (1977) states that the human urothelium in a moderately dilated bladder is composed of approximately seven layers of cells but it is probable that the biopsy
examined in that investigation relating to tumours, came from a hyperplastic area. In a recent paper, Knowles et al. (1980) who examined bladder mucosal biopsies from patients of widely varying age have reported that the normal urothelium is not more than five cell layers thick. Tannenbaum (1979) has shown electron micrographs of normal human urothelium with two to three layers of intermediate cells which would make the urothelium four to five cell layers thick in all. In the young adults examined in the present study, two or more layers of intermediate cells occur and it is rather difficult to be certain about the exact number of cell layers present in the urothelium. This uncertainty is further accentuated by the variable state of distension or contraction of the bladder as well as the plane of sectioning. These considerations can explain the apparently six cell thickness of the urothelium observed sometimes in the young adults. The observations of most workers hitherto put the thickness of the normal human bladder urothelium in the range of three to four or four to five cell layers. In their study of the urinary bladder in children aged two to thirteen years, Haynes et al. (1975) could not determine accurately the thickness of the urothelium because of the tangential sectioning of the tissue; they were only able to say that no fewer than three cell layers were present in any normal specimen. During embryonic development of the human foetus, the bladder urothelium becomes three cells thick by about the fifth or sixth month, but there is evidence of differentiation characteristic of the adult urothelium by the end of the third month when the urothelium is only two cells thick (Hoyes et al., 1972). By the fifth or sixth month of pregnancy, the essential features of the human bladder urothelium would appear to be present with basal cells (resting on a basal
lamina), intermediate cells, and differentiating superficial cells lining the lumen. In the literature, there is a singular lack of data from infants and young children on whether the three cell thickness reached in the foetus is strictly maintained or whether the urothelium becomes thicker later by the formation of additional layers of intermediate cells. We know from the work of Haynes et al. (1975) that, in children aged two to thirteen years, the urothelium contains a minimum of three cell layers but they were unable to establish the maximum number of layers that may have been present in the specimens they examined, because, as explained above, the sections were cut tangentially. However, in the light of the present observations in young adults, it would seem likely that the urothelium of the foetus probably increases in thickness in young children, and thereafter remains stable with a maximum of five cell layers.

Hyperplastic urothelia with six to ten or more cell layers have been described by Knowles et al. (1980), but such proliferative lesions were not met with in any of the specimens that were examined in the present study. There were also no indications in the stroma, suggestive of inflammation in any of the specimens. The present findings on the varying numbers of cell layers in the urothelia of the different age groups further suggests that while the thickness of the tissue remains more or less constant in the young adult and the middle aged groups, it becomes noticeably thinner in the aged subjects. That the urothelium of the elderly may contain fewer cell layers than that of the adult was indicated by Jacob et al. (1978) in their study limited to elderly patients. My investigation, which has included younger age groups, confirms their observation and shows that the unusually thin regions that were thought of as
characteristic of aged urothelia may occur in subjects only fifty-eight years old. From the present work, it would also appear that the frequency of the occurrence of thin urothelial regions in the elderly is greater than what was indicated by Jacob et al. (1978).

An aspect of great uncertainty in the literature on human urothelium is that relating to the surface of the cells lining the lumen of the bladder. The early investigators (Battifora et al., 1964; Monis and Zambrano, 1968; Flaks et al., 1970) reported the presence of microvilli on the free luminal surface. However, Hicks (1965, 1966a, b) who investigated the urothelium of the rat reported that the luminal surface was ridged and angular. In their description of the ultrastructure of the normal bladder urothelium of man, Fulker et al. (1971) observed that the luminal membrane was raised into microvilli sometimes, but not always. From a careful reading of their paper, it is obvious that the luminal surface was not uniform in appearance and that in parts it was microvillous and in parts it was angular with a specialised plasma membrane. This could explain the discrepancy between some of their transmission and scanning electron microscope observations which they probably made without realising the variability of the surface. Later, Firth and Hicks (1973) examined the bladder urothelium of sixteen species of mammals, including man, and described the presence of a thick angular luminal plasma membrane in all cases. The only human bladder material available to them was admittedly unsuitable as the superficial cells had undergone necrosis or post-mortem autolysis and no microvilli were observed in the restricted fragments they examined. In negatively stained preparations of scrapings of human bladder urothelium obtained after death, Warren and Hicks (1973) were able to demonstrate a substructure similar to that of the uniquely specialised angular luminal
membrane of the rat and other mammals investigated. There is no
doubt, therefore, that specialised membrane regions are present in
the luminal surface of the human bladder urothelium, but what extent
of the surface is limited by such a membrane has not so far been
examined critically. In animals, particularly the rodents, it is
clear from repeated investigations (Walker, 1960; Leeson, 1962; Richter
and Moize, 1963; Hicks, 1965, 1966a,b; Koss, 1969) that the specialised
membrane is very prominent and probably 73% to 90% of the surface is
limited by such a membrane. During the period when data were being
accumulated in animals, there was practically no systematic or thorough
study of human tissue due to the difficulty of obtaining specimens.
The evidence derived predominantly from animal studies appears, there¬
fore, to have led to the generalisation (cf. Hicks, 1975 and others)
that the specialised luminal membrane is as prominent or extensive in
the human urothelium as it is in the rat. The observations from
human material were fragmentary and incomplete as stated above, and
the attempt to fit the scanty data from humans with that observed
widely in non-primate mammals appears to me to have been premature.
The evidence from study of animals was so overwhelming that sporadic
observations from human urothelium about the occurrence of flexible
luminal membrane and microvilli, at least in parts of the luminal
surface of the bladder and ureteric urothelium (Battifora et al.,
1964; Monis and Zambrano, 1968; Flaks et al., 1970; Fulker et al.,
1971) were regarded as erroneous interpretations (Firth and Hicks,
1973) arising from the loss of differentiated superficial cells
during preparative procedures. The microvilli were thus assumed to
be those of the undifferentiated cells of the intermediate layers which
were accidentally exposed to the lumen following the loss of the superficial
cells.
In recent years, with more efforts to obtain and study the human bladder, there have been several reliable reports of the presence of microvilli on the free luminal surface of normal urothelia. Merk et al. (1977) described uniform but variable numbers of microvilli at the luminal surface in adults; the vast majority of the luminal surface was reported to be smooth and microvillous but specialised angular membrane regions with asymmetric structure were not totally absent; they were only relatively rare. In contrast, they found that, in infants, the majority of the luminal surface was limited by the specialised plasma membrane. The abundance of the specialised membrane in the luminal surface of the urinary bladder of newborn humans has also been pointed out by Alroy et al. (1977). At the same time, they also reported that the luminal surface of the adults was almost entirely devoid of such specialised membrane; an electron micrograph presented in their paper (Fig. 1; Alroy et al., 1977) clearly shows microvilli at the surface.

References to microvilli coexisting with microridges on the luminal surface of human bladder, or references to specialised membranes being not so prominent in man as they are in rodents, can be found scattered in the literature (cf. Cooper, 1972; Pauli et al., 1978; Tannenbaum et al., Alroy and Gould, 1980; Alroy et al., 1981). Knowles et al. (1980) observed small microvilli or blebs in human urothelia - on the luminal surface of "otherwise normal superficial cells". But they seem to hold the view that microvilli are an abnormal feature somehow associated with urinary obstruction or benign prostatic hypertrophy of the patients. There is evidence from the work of Tannenbaum et al. (1978) and others that the cytoplasm of superficial cells in patients with prostatic hypertrophy is as well differentiated as in the rodent. In the present state of our knowledge,
therefore, one cannot subscribe to the view of Knowles et al. (1980) that prostatic hypertrophy or bladder neck obstruction invariably causes failure of urothelial differentiation and normal maturation. My observations suggest that the presence or absence of microvilli is more probably related to the age of the patient. As mentioned before, during embryonic development, microvilli appear first before angularity (specialisation) of the surface membrane develops (Hoyes et al., 1972). A careful study of the literature clearly shows that the specialised membrane, appearing as characteristic asymmetric unit membrane plaques on the luminal membrane, or as discoidal vesicles in the superficial cell cytoplasm, is never as prominent or abundant in man as in the other species of mammals investigated, especially rats. There are reports in the literature that, in humans, the asymmetric unit membrane is best seen during childhood (Alroy et al., 1977; Merk et al., 1977). However there is an anomalous report (Haynes et al., 1975), where the authors state that in children, the luminal membrane is angular and about 12 nm thick, yet that this membrane, which was thicker than the plasma membrane of other cells, was symmetrical in structure. Generally, and in man, it is the asymmetrical unit membrane which is 12 nm thick while the symmetrical membrane is 10 nm thick (Alroy et al., 1977; Alroy and Gould, 1980 and others). It would hence seem, that the thicker luminal membrane observed by Haynes et al. (1975) in children aged two to three years was really asymmetric but was inadequately examined or insufficiently resolved in their preparations. In the present investigation, my finding is that in young adults also, a sizeable area of the luminal surface is covered by asymmetrical membrane. Together with the observations of Alroy et al. (1977) and Merk et al. (1977) this would extend
the period of the most widespread occurrence of asymmetrical unit membrane in the luminal surface of the human bladder from birth to about thirty years of age. Even though the luminal membrane is asymmetrical, a point of interest is that the luminal surface generally has a smooth or at most, a wavy contour, quite unlike the typical angular formations of the asymmetric unit membrane plaques seen in rats. Moreover, there are not many of the typically fusiform or discoidal vesicles in the apical cytoplasm of the superficial cells as in rats; instead, the many vesicles present are either spherical or elongated, suggestive of flexibility rather than rigidity of the limiting membrane.

In the age group which has been referred to as the middle-aged in the present study (subjects aged 35 to 49 years), the luminal surface displays both symmetric and asymmetric types of membrane. However, it is again noteworthy that in regions which appear scalloped, the luminal membrane may be either symmetric or asymmetric in structure. It is only in the regions exhibiting microvilli that the membrane is invariably symmetric. Indications in the human bladder urothelium then are for an apparent lack of rigidity in the asymmetric unit membrane. This view is supported by the lack of many typical fusiform vesicles in the cytoplasm even though there are a number of cytoplasmic vesicles limited by asymmetric unit membrane.

In elderly subjects, the luminal surface is generally microvillous in both the thin and thick regions of the urothelium; the microvilli are short and sparsely distributed. The cells of the thin regions show an unremarkable ultrastructure, being as undifferentiated as the basal cells in bladder urothelia generally; the luminal plasma membrane of these cells would therefore be expected to be of the non-specialised
type. The luminal plasma membrane of the superficial cells in the thick regions are almost exclusively of the non-specialised type exhibiting a symmetrical structure. This was also the structure of the membrane limiting the free vesicles in the apical cytoplasm of the superficial cells.

Results of the careful examination of the luminal surface of urothelium from different age groups in the present study, together with the data from young children available in the literature, suggest that there is a progressive decline in the extent of the specialised asymmetric plasma membrane from infancy to old age. The uniquely specialised membrane which covers the major part of the bladder lumen in rats and other rodents certainly exists in man and it is only its extent and degree of manifestation that are in question. The frequency of the specialised membrane is highest during childhood but even here, it is not so remarkably prominent as in the rat. The young adult and the middle-aged show a lower frequency but in the aged, the production of such a membrane appears to have practically ceased. The possibility of such an age-related decline, foreshadowed by two groups of workers independently (Alroy et al., 1977; Jacob et al., 1978), has now been confirmed. That structural and chemical differences may occur in specialised plasma membrane components of certain other young and old human cells has been demonstrated before (Marikovsky et al., 1976; Schekman et al., 1976). Changes have also been reported in the specialised membrane components of various cell types, including human, during in vitro ageing (Kelley, 1976; Azencott et al., 1975).

Apart from the ultrastructural differences described so far in human bladder urothelia of different ages, the basic features of the
urothelium in man are similar to those in other species of mammals. There is no doubt that there are three morphologically different types of cells and that when in a distended bladder, more than three cell layers are found, the increased thickness is due to the increased numbers of intermediate cell layers. The lateral movement of cells in and out of the intermediate layer during bladder contraction and distension as originally pointed out by Richter and Moize (1963) has also been generally accepted. The basal cells are attached to the basal lamina by hemi-desmosomes but the cells of the intermediate and superficial layers are normally not in contact with the basal lamina which separates the lamina propria from the epithelial cells. Recently, doubts have been expressed whether human urothelium represents a true stratified or a pseudo-stratified epithelium (Knowles et al., 1980). This aspect was examined in the present investigation and it can be stated with some certainty that in the adult and middle-aged urothelia examined, there were no instances where the superficial cell or its surface processes contacted the basal lamina. The bladder urothelium is hence a true stratified epithelium of the transitional type as generally understood by these terms. However, in aged subjects, the appearance of the urothelium can be deceptive in places. In the thin regions (one and/or two cells thick) and in the junctional regions between the thin and thick regions, the urothelium may appear to be simple and/or pseudo-stratified. Such regions have not been observed in the normal urothelia of the rodents and other short lived species of mammals.

In the course of this study of human urothelium from different age groups, there have been instances where very electron dense or dark cells were found amidst clear ones at all levels. It may be of
some significance that dark cells were met with more often in the elderly urothelium than in the younger specimens. The mitochondria of the dark cells were sometimes swollen. Several investigators of human urothelium have noted dark cells in normal bladder as well as in urothelial tumours (for example: Kasiwai et al., 1963; Fulker et al., 1971; Tannenbaum et al., 1978; Jacob et al., 1978). Such cells have also been noted in human foetal bladder by Hoyes et al., (1972) who suspected that its frequency was higher in tissue fixed by immersion than in perfused foetuses, but the significance of the dark cell phenomenon remains unclear. In the literature, there are no reports of dark cells in the normal urothelium of rats and other animals. However, Koss (1977) observed dark cells in the urothelium of rats four months after the initiation of a carcinogenic diet, when there was clear evidence of necrosis. Likewise, Raick (1973) observed dark cells in epidermis of adult mice treated with a tumour-promoting substance; the dark cells are also reminiscent of similar cells seen in foetal or perinatal mouse epidermis as well as in epidermal tumours (Mufson and Weinstein, 1981), where death of individual cells could be expected to occur. In view of the fact that dark cells occur in the aged urothelium with a greater frequency than in the younger urothelia and in view of the suggested correlation of dark cells with necrosis or with cell death in the course of embryogenesis or in tumours, it is tempting to make the speculation that the cells in question are deteriorating or damaged and that their life span will be limited. More evidence will, however, be required to confirm this.

It is known that a slow turnover of cells occurs naturally in mammalian urothelia when the desquamated or exfoliated superficial
cells are replaced by cells from the layer immediately below (Cooper, 1972; Martin, 1972; Firth and Hicks, 1973). Urinary bladder epithelial cells are known to exfoliate in all mammals and are frequently seen in microscopic analysis of urine of humans, but little is known about the longevity of urothelial cells in the human bladder. In rodents as well as in dogs and cats, the urothelium is very stable and mitoses are seldom seen in the adult bladder. In the present study also, I have not come across dividing cells and this suggests that mitosis is infrequent in normal human urothelia of the different age groups examined. Autoradiographic studies in the mouse following the use of radioactive thymidine have shown that the proportion of cells synthesizing DNA is between 1.0% and 0.5% and it has also been estimated that urothelial cells have an average life span of 50 to 200 days (Levi et al., 1969; Dzhioev et al., 1969). In the rat, the labelling index was between 0.2% and 0.5% (Messier and Leblond, 1960; Locher and Cooper, 1970). Evidence from the investigations of Messier and Leblond (1960) and Martin (1962, 1968, 1972) in rat and guinea pig show that mitoses occur mostly in the basal layer of the urothelium. However, in mouse urothelia regenerating rapidly from acute damage, mitoses have been observed (Walker, 1959; Levi et al., 1969) in all three layers of cells. In the absence of data on cell proliferation kinetics in human urothelium, and in analogy with other lining epithelia, one may assume that cell division in normal undamaged urothelium is confined to the basal layer of cells. Lipkin (1971, 1973) has shown for example that during migration of normal colonic epithelial cells through the lower-middle third of the colonic crypt, DNA synthesis and mitoses cease as the cells undergo differentiation.

As mentioned above, a number of investigators have shown that the
turnover rate of cells in normal bladder urothelium in mammals is low and that their lifespan is long, although the urothelium is well known (cf. Cooper, 1972; Hicks, 1975) for its capacity to recover from acute damage or injury by rapid cell proliferation. In the guinea pig, it has been estimated that following mild trauma, in the form of overdistension of the bladder, when there is active proliferation, it takes more than eleven weeks for a cell to migrate from the basal layer to the superficial layer (Martin, 1972), whereas after more acute damage, like peeling off or denudation of urothelium, the re-establishment of a normal differentiated urothelium was reported to take four to five weeks (Hanson et al., 1969). In normal circumstances then, the vertical migration of cells and the process of maturation may be expected to take a much longer time, and this may hold true for human urothelium as well. Some idea of the time-scale may be had from observations on human bladder urothelium recovering from various forms of damage; regeneration has been estimated to take six weeks (Lund, 1969), six months (Likourinas et al., 1979), or ten to twelve weeks (Jacob et al., 1982). In these investigations, the criterion for the restoration of normal urothelium was the replacement of a denuded urothelium with a hyperplastic one, whereas strictly speaking, normality would be when a topographic distribution of urothelial cells into superficial, intermediate and basal is attained and the urothelium exhibits a pattern of progressive cytodifferentiation.

It is well documented in the literature that cells in the basal layer arising presumably from stem cells serve as replacements for the overlying intermediate and superficial layers, and that during the long period of migration to the surface, the cells gradually
differentiate. This process of gradual cytoidifferentiation has been found in the young adult and middle aged urothelia where it may be assumed that the urothelium exists in a state of dynamic equilibrium with a balance between cell proliferation in the basal layer and the loss of cells from the luminal surface allowing for the long lifespan of the superficial cells. An explanation for the gradual decrease in the thickness of the urothelium that was noted with increasing age and finally, the appearance of urothelium or stretches of it, only one cell thick or occasionally even denuded to the basal lamina in the elderly, may be sought in an imbalance in the rates of cell proliferation and cell loss and hence in the loss of the steady state mentioned above. One might therefore speculate that, with increasing age, there is a reduction in the rate of proliferation of stem cells in the basal layer while the rate of desquamation from the surface remains the same or perhaps increases. In this context, it may be significant that the frequency with which dark cells are met with is highest in the urothelium of the elderly. If these cells are dying cells, as suggested earlier, it would lend support to the view that desquamation is a little more frequent in the aged urothelium than in the younger tissue.

It is known that in stratified epithelia, the basal layer of cells contains a proliferative pool of cells, the so-called stem cells, and that this pool is maintained throughout the life of the organism. Newly formed cells resulting from mitotic activity move to the cell layer above and differentiate as they migrate vertically to the superficial layer. Normally, the rate of proliferation of stem cells is strictly controlled in that they divide just enough to replace cells lost from the superficial layer. In certain conditions, however,
such as during regeneration following damage or injury to the tissue, the rate of proliferation can increase. On the other hand, there are other tissues such as muscles and neural tissue where stem cells probably cease to exist after embryogenesis. We do not as yet understand the molecular basis of ageing in cells, but there is a point of view that the cells that remain relatively unspecialised and continue to divide do not age, or age more slowly than differentiated cells which have lost the capacity to divide.

In cultures of several human diploid somatic cells with initially high proliferative capacity it has been reported that they tend to decline because of a progressive loss of cells in the population which are capable of division (Hayflick and Moorhead, 1961; Merz and Ross, 1969) and that clones of such cells eventually cease proliferating (Martin et al., 1975). These workers have put forward two alternative theories to explain the phenomenon of clonal senescence, but what is important in the present context is the probable occurrence of this phenomenon in vivo. We do not know the total division potential of the urothelial stem cells in vivo, but one may speculate that, with increasing age, there is a progressive loss of cells in the stem cell population which are capable of undergoing cell division, similar to the decline observed in cultured diploid cells. Although the artificial environment of cell culture is substantially different from the in vivo environment, the analogy provides a basis for speculating that with age, there is a progressive decline in the number of stem cells undergoing division, and that, it is as a consequence of this that the urothelium in the elderly is thinner than in the young. The basis for this decline in the proliferative capacity of old cells mentioned above is not known and the suggestion is that it is an intrinsic property of the ageing
process (Hayflick, 1965).

Another finding that has emerged from the present study of human bladder urothelium of different age groups that requires further discussion is the changing nature of the luminal surface. Before embarking on this, however, what needs to be stressed is the fact that AUM plaques are not so characteristically prominent in the humans as in the rodents, particularly rats. Even in the youngest age group examined in this study and in children (reported in the literature), where the specialised luminal plasma membrane is probably best visualized during the entire human lifespan, my impression is that it is not by any means as extensive as it is in the rat. Moreover, the AUM, where it is present either on the luminal surface or in the cytoplasmic vesicles of human bladder urothelial cells does not appear to be as rigid as in rat. This aspect will be discussed later. The relative scarcity of AUM on the luminal membrane in humans is matched by a parallel observation which tends to confirm this observation. That is, the AUM-limited vesicles in the cytoplasm of the superficial cells which serve as reservoir AUM for the luminal surface are also relatively scarce in humans compared to rats.

It is now well established that the AUM is formed within the Golgi complex (Hicks, 1966b, 1975; Koss, 1969) of the superficial urothelial cells mainly. During the present investigation, not many instances of AUM associated with the Golgi apparatus were met with. It is probably owing to the relative scarcity of AUM in the urothelium of humans that it has been rather more difficult to demonstrate the association of AUM with the Golgi apparatus in humans (Jacob et al., 1978). The Golgi apparatus in the superficial cells of the young adult is extensive and well developed: these cells have several vesicles in
the apical cytoplasm but not all of the vesicles are limited by AUM.
The point of interest is that not all of the plasma membrane-bearing vesicles produced by the Golgi apparatus in this well differentiated cell are limited by AUM; in other words, the presence of several SUM-limited vesicles suggests that the Golgi apparatus in the young urothelium is involved not only in the production of AUM but also SUM, perhaps more of the former than the latter. In the middle aged group, the Golgi apparatus in the superficial cells is also extensive and well developed as in the young. The appearance of the luminal surface of this group is variable as explained earlier and a subjective interpretation is that there is more of the SUM at the luminal surface and more of SUM-limited vesicles in the apical cytoplasm of the superficial cells than in the younger urothelium. It seems likely then that more of the symmetrical type of membrane is formed within the Golgi complex of the differentiated superficial cells of the middle-aged urothelium than in the Golgi of the differentiated superficial cells of the younger urothelium. In the elderly subjects, the superficial cells of the thick region of urothelium possess well developed Golgi complexes with stacks of cisternae and vesicles of various sizes. The luminal plasma membrane of these cells appears to be generally flexible and typical AUM plaques are practically absent. These findings confirm previous observations by Jacob et al. (1978) in a group of elderly patients. Occasionally, however, they found isolated instances of fusiform vesicles bounded by AUM in the apical cytoplasm of some of the superficial cells. From these observations, it seems reasonable to conclude that if AUM plaques and AUM-limited vesicles occur at all in aged urothelium, they must be very rare. Since well developed Golgi complexes are present in the
cells concerned, one might speculate that in the aged urothelia, the rough endoplasmic reticulum and Golgi apparatus do not function in the assembly of the asymmetrical type of plasma membrane but of symmetrical membrane. This implies that, compared with the young urothelia, there has been an alteration in the nature of the membrane synthesised in the aged urothelia. The luminal plasma membrane is largely symmetrical in structure and many of the luminal cell junctions are probably abnormal and hence permeable. These features might mean increased permeability and less adaptability of urothelial function to mechanical stress caused by changes in bladder volume. In this context, it should be pointed out that the urothelium of aged rats much nearer to the end of their natural lives than that used in the present work remains to be examined.

The unusual structure, chemical composition and molecular organisation of the AUM obtained from the bladder urothelia of various species of mammals have been investigated by a number of workers using a variety of techniques such as negative staining and freeze fracture. Although a considerable volume of information has been obtained (see review by Hicks, 1975), it is not yet clear whether it is the proteins, lipids or carbohydrates that contribute to the ultrastructural asymmetry of the specialised membrane; much therefore remains to be investigated about the molecular organisation of this asymmetrical membrane. Perhaps as much effort should be put into investigating this membrane as had been deployed to investigate the erythrocyte membrane. All known biological membranes are asymmetric in that the inside and outside faces of membranes are different and in the case of plasma membranes, this is exemplified
generally by the external location of membrane carbohydrates. It has been known for some time (Singer, 1974) that components of biological membranes are disposed asymmetrically across the membrane, and direct evidence is available to confirm the asymmetry at the molecular level; that is, asymmetrical distribution of protein components and perhaps also the phospholipids of the erythrocyte membrane. Apart from the AUM of the urothelium, other examples of morphologically asymmetrical plasma membrane with either the outer or the inner leaflet of the unit membrane being morphologically thicker are available in the literature (cf. Hicks, 1965; Mollenhauer et al., 1977).

The apical surface of many differentiating cells is known to amplify, and a well-known example is the differentiation of the brush border at the apical surface of intestinal absorptive cells. In *Xenopus* where brush border formation has been investigated in detail, Bonneville and Weinstock (1970) have shown that special surface-forming vesicles arise in the Golgi region, move subsequently to the apical region of the cell and finally get incorporated into the free cell surface. The plasma membrane at the free surface expands by the incorporation of pre-formed units of membrane rather than through any process of self-assembly of molecules at the surface itself. Much the same sequence of events was shown to occur in the incorporation of asymmetric unit membranes into the luminal plasma membrane of superficial cells of rat bladder urothelium by Koss (1969).

There is a large body of evidence that the plasma membrane of cells is largely assembled internally and then transported to the surface. The plasma membrane is hence pre-formed rather than formed
epigenetically. Evidence from electron microscopic and biochemical investigations have shown that the plasma membrane is pre-formed as a result of an orderly series of events mediated by ribosomes and Golgi complex (Morré et al., 1974; Whaley and Duwalden, 1979; Lewis, 1980). The plasma membranes owe their specific properties to membrane proteins produced by polysomes (mainly membrane-bound) or to membrane glycoproteins or glycolipids produced in the endoplasmic reticulum and Golgi complex. No protein synthesis is believed to occur in the Golgi apparatus but it can synthesize oligosaccharides from simple sugars and attach them to proteins and lipids to form glycoproteins and glycolipids. The Golgi apparatus mediates in glycosylation - actually terminal glycosylation - (since some core glycosylation can take place in the rough endoplasmic reticulum), and thereby confer the plasma membrane proteins passing through it with key characteristics. The role of the Golgi complex in membrane biogenesis is then as an intermediary station where membrane may be modified, while in transit from the endoplasmic reticulum to the cell surface. In other words, some key characteristics of the plasma membrane are given to it by the Golgi apparatus by the addition of sugar chains to the protein component of the membrane. As explained before, the AUM of the bladder urothelium is ostensibly derived from the Golgi complex and on the basis of our current knowledge of the plasma membrane formation, the special characteristics of the AUM may be acquired in the Golgi complex. If so, the decline or failure of AUM production in ageing human urothelia may be ascribed to a failure of the Golgi apparatus to produce some specific polysaccharide. One cannot, however, rule out the possibility that the practical absence of AUM in the aged urothelium may be related to a failure of
synthesis of specific proteins or phospholipids (the main constituents of membranes); this would be a failure of gene expression at an earlier or primary level. But, as pointed out by Whaley and Duwalder (1979), the glycosylation activities of Golgi apparatus are also probably under genetic control—a later stage or a secondary level in the expression of genetic control—in that the glycoprotein synthesis may be controlled by the availability of enzymes and/or the specificities of individual glycosyl transferases. The practical absence of the thick, asymmetric, cell membrane segments in the superficial cells of the aged urothelium may therefore be viewed as some sort of functional failure or alteration of gene expression. The Golgi complex may be the site of this failure, but further knowledge about the nature of the asymmetry at the molecular level should help to clarify this question. An insight into the nature of the suggested functional alteration or failure of differentiation is provided by the result of other workers who have shown how AUM production can be influenced and modulated by external agents. By maintaining rats on a diet deficient in essential fatty acids, Monis et al. (1976) were able to reduce the overall thickness and ultrastructural asymmetry of the AUM at the luminal surface and in the large vesicles in the apical cytoplasm; these changes were reversed on restoring the fatty acids to the diet. On the basis of these observations, they suggested that the unusual asymmetry of the membrane is due to its lipid components rather than its proteins and that the lipids may have an asymmetric arrangement in the molecular organisation of the membrane as has been shown by Bretscher (1973) for phospholipid and glycolipid components in red blood cells. In the bladder urothelium of the human adult, Alroy et al. (1977)
observed an increase in AUM plaques after incidental exposure to therapeutic X-irradiation and they attributed this increase to a stimulation of the Golgi complex by X-rays.

The biogenesis of plasma membrane must obviously be an ongoing function of urothelial cells, and as is now recognised (see Tartakoff, 1980), the membrane components are only one of the three classes of biosynthetic products that pass through the Golgi complex from the rough endoplasmic reticulum; the others are secretory products and lysosomal proteins. The Golgi complex of human urothelial cells has been demonstrated to be involved in the production of lysosomes (Jacob et al., 1978), and a clear topographical association of Golgi apparatus with lysosomes has been noted before in the rodent urothelium (Hicks, 1966b; Koss, 1969). In the superficial cells, it has also been pointed out (Jacob et al., 1978) that acid phosphatase-containing vesicles of Golgi complex move to the apical cytoplasm and fuse with the luminal membrane. The bladder urothelium is generally assumed to be a non-secretory epithelium and what merits consideration now is the transport of the plasma membrane components from the Golgi complex to the cell surface. The involvement of a class of fairly large vesicles in the delivery of the asymmetric type of membrane to the luminal surface of the urothelium is already documented (Hicks, 1966b; Koss, 1969), but this leaves open the question of the transport of the symmetric type of plasma membrane to the cell surface. The present investigation shows that the thick, asymmetric type of plasma membrane is not so prominent in humans as in rodents, and that the production of such membrane tends to decline as the urothelium ages. This is reflected in the observation that the luminal surface in the
young and middle-aged urothelia is made up of varying proportions of asymmetrical and symmetrical unit membranes and that finally in the elderly subjects the luminal surface is virtually free of the asymmetric membrane. This, in turn, is linked with the presence, in varying proportions, of large vesicles lined by AUM or SUM in the apical cytoplasm close to the luminal membrane in the first two age groups and the practical absence of AUM-limited vesicles in the old. The polarisation of large vesicles, more or less similar in size and also shape, as demonstrated in this study, makes it likely that the delivery of plasma membrane from the Golgi complex to the luminal surface specifically is accomplished in a class of large vesicles. These vesicles remind one of the surface-forming vesicles directed to the apical surface in differentiating intestinal absorptive cells of *Xenopus* (Bonneville and Weinstock, 1970). Further support for the assumption made above comes from experimental studies in rat. In regenerating urothelium where the luminal surface of superficial cells was covered by symmetrical membrane, it was found that the apical cytoplasm contained large vesicles limited by SUM. Later when AUM plaques appeared at the luminal surface, large vesicles with asymmetrical limiting membrane appeared beneath the surface.

Another component noticed in the Golgi complex of the urothelial cells is the population of small, coated vesicles; as described in the text, some of these appeared to be fusing with or budding from other Golgi elements. In the literature, coated vesicles have been described in a variety of cells but their precise role is unclear. There is now general agreement that the Golgi complex is involved in the processing of a wide range of secretory products, characteristic of different cell types, that it is involved in the production
of lysosomal proteins, and that it contributes to the formation of the plasma membrane. It is also becoming clear that the pleomorphic system of membranes that comprise the Golgi complex consists of multiple sub-compartments with possibly differing functions (Tartakoff, 1980). Koss (1969) has also speculated that a new area of the Golgi complex may become activated in superficial cells for the production of AUM. Coated vesicles with diameters in the range 50-150 nm have been reported in several cell types, and they have been ascribed a role in endocytosis (Friend and Farquhar, 1967; Goldstein et al., 1979) and in membrane recycling in cells (Pearse and Bretscher, 1981). The pinocytotic activity noticed along the lateral border of superficial and other urothelial cells in the present study, as well as in previous publications (Haynes et al., 1975, Tannenbaum, 1979), may well be related to endocytosis from the cell surface (centripetal traffic) involving coated vesicles. The endocytic activity is probably a mechanism for taking up special proteins into urothelial cells from the extracellular compartment of the urothelium which is open at the level of the basal lamina and hence in communication with the sub-epithelial lamina propria.

Biochemical and kinetic evidence now available (Rothman and Fine, 1980) show that coated vesicles may serve to carry plasma membrane from Golgi apparatus to the cell surface. This may be the case in cells generally, and in the urothelial cells, they may carry plasma membrane from a sub-compartment of Golgi complex to the lateral surfaces. The pathway, perhaps from another sub-compartment of the Golgi complex, of plasma membrane destined for the luminal surface of the superficial cells (whether symmetric or asymmetric) would then appear to be different from that taken by plasma membrane for the other surfaces. There is, in fact, some
evidence for distinct intracellular pathways for centrifugal transport of different classes of products (Strous and Lodish, 1980; Gumbiner and Kelly, 1982). This specificity is similar to that exhibited by secretory vesicles, for instance, the secretory vesicles of the pancreatic acinar cells, where they fuse only with the apical portion of the plasma membrane; the molecular mechanism involved in this type of specificity is unknown.

In the mammalian urothelium, it is well established that there is a progressive increase in size of the cells from the basal to the superficial layer. The quantitative analyses in this study show that in the young adult and middle-aged urothelia, the superficial cell is six times larger than the basal cells and about three times larger than the intermediate cells. It is only in the aged urothelia that such a large sized superficial cell is not met with. The superficial cells in the thick regions of the aged urothelium are significantly smaller than those in younger urothelia. But, it is interesting to note that the nuclei of those relatively small superficial cells of the aged urothelium are of much the same size as their counterparts in younger urothelia. The smaller size of the superficial cells in the elderly is then due to the smaller size of their cytoplasm.

The superficial cells under discussion, though somewhat small, are still about four times larger than the basal cells and the most differentiated of the urothelial cells. The present study confirms the existence of a pattern of progressive cytodifferentiation from the basal to the superficial layer in the thick region of the urothelium of the elderly, as was reported by Jacob et al. (1978). Another noteworthy feature that has emerged for the present morphometric
study is the uniformity in size of the nucleus and the overall size of
the cells of the basal layer in all the three age groups.

During the course of this investigation, binucleate cells were
met with only very infrequently. An occasional one was noted in the
youngest age group but practically none in the old age group. In rat,
mouse and rabbit, some workers have reported multinucleated super-
ficial cells and they (Levi et al., 1969, 1971; Cooper, 1972;
Hicks, 1976) hold the view that superficial cells are generally poly-
ploid. In the guinea pig (Martin, 1972), the intermediate cells are
believed to be produced by fusion of basal cells and that the inter-
mediate cells, in turn, fuse to produce large, polyploid superficial
cells; it is also held that, since these cells are formed primarily
by fusion, the initial ratio of nucleus to cytoplasm is maintained.
These observations are contrary to the earlier observations of
Kanczak (1964) in rodents, such as rat, mouse and hamster and
therefore the question of polyploidy in the superficial cells of
the mammalian urothelium remains to be clarified. There is no
evidence of cell fusion in the human urothelia of any group examined
in this study and there is no evidence of cell fusions in early stages
of development of the human foetal bladder (Hoyes et al., 1972).
The present morphometric data show that the nucleo-cytoplasmic ratio
is anything but constant from the basal to the superficial layer,
and do not lend support to the view that a process of cell fusion
is involved as the cells migrate vertically from the basal layer or
that the superficial cells are polyploid. In a computer study of
normal human urothelial cells, Koss et al. (1975) could find no
evidence of polyploidy but the large size of the superficial cells
has been taken by some investigators of human urothelia (Fulker
et al., 1971; Knowles et al., 1980) to be indicative of polyploidy. Only careful cytophotometric investigations in the future can resolve this problem.
CHAPTER III

EXPERIMENTAL INDUCTION OF BLADDER TUMOUR IN RATS

Introduction

The first demonstration that bladder tumours could be chemically induced in experimental animals was made by Hueper and co-workers (Hueper and Wolfe, 1937; Hueper et al., 1938), who observed bladder carcinoma in dogs fed or injected with 8-naphthylamine. This chemical can also produce bladder carcinomas in hamsters, monkey and possibly rat (Bonser et al., 1952; Sellakumar et al., 1969; Hicks et al., 1976). Many other aromatic amines have been investigated, and the literature up to 1970 was reviewed by Clayson and Cooper (1970), who have provided an extensive bibliography. Of the compounds investigated, 2-acetylamine fluorene (2-AAF), which had been produced as a possible insecticide, proved to be a potent bladder carcinogen for rodents as well as dogs, and has been extensively used as an experimental tool in many laboratories (Wilson et al., 1941; Oyasu et al., 1963; Melicow et al., 1964).

Another widely used compound was the nitrofuran derivative N-[4 - (5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT). It was found to be a potent bladder carcinogen in rat (Erturk et al., 1969), mouse (Erturk et al., 1970a) hamster (Croft and Bryan, 1973) and dog (Erturk et al., 1970b), but all species developed tumours in other organs as well. An unrelated compound, the sulfonamide ENS, has been much used by the Leeds group of investigators as a bladder carcinogen in mouse (Clayson and Bonser, 1965; Clayson et al., 1967).

After the discovery that dimethyl nitrosamine was acutely hepatotoxic to a number of laboratory animal species (Barnes and Magee, 1956), tests
for its possible carcinogenic action were carried out and it was shown that nearly all rats on a diet containing 50 ppm of this chemical developed malignant liver tumours within less than a year (Magee and Barnes, 1956). The first chemical carcinogen identified as a specific for rat bladder was butyl-butanol-4-nitrosamine when Druckery and Müller (1962) showed that continuous feeding with this compound was 100% effective in producing bladder tumours. A very large volume of literature has now accumulated on the carcinogenic activity of a whole range of nitroso compounds, reviewed by Druckery et al. (1967) and Magee and Barnes (1967). So far, nitrosodibutylamine (DBN), butyl-4-hydroxybutyl nitrosamine (BBN), N-butyl-N-(3-carboxypropyl)-nitrosamine, N-ethyl-N-(4-hydroxybutyl)-nitrosamine, nitrosomethyl-dodecylamine and N-methyl-N-nitrosourea (MNU) have been shown to be potent inducer of urinary bladder tumours in rodents.

DBN was found to produce bladder tumours in rat (Druckery et al., 1962) mouse (Bertram and Craig, 1970; Wood et al., 1970); guinea pig (Ivankovic and Buchler, 1968), and Syrian and Chinese hamster (Mohr et al., 1970), but tumours developed at other sites also in all species. The potency and specificity of BBN as a bladder carcinogen has been confirmed in rat (Ito et al., 1969), mouse (Bertram and Craig, 1972), and hamster (Althoff and Krüger, 1975). However, Hirose et al., (1976) have reported that BBN failed to produce bladder tumours in guinea pigs. N-ethyl-N-(4-hydroxybutyl) nitrosamine has been shown to be a potent bladder carcinogen in rat, but less effective in mice and hamsters, although the bladder carcinomas in mice were very invasive (Hirose et al., 1976). Nitrosomethyl dodecylamine has been reported to be a bladder carcinogen for Sprague-Dawley rats, but it also produces tumours in the lung and oesophagus (Lijinsky and Taylor, 1975).
MNU is carcinogenic for a number of organs of several species with the tissue specificity depending upon the route of administration - orally or by injection - and the dose schedule (Druckery et al., 1964; Leaver et al., 1969; Schreiber et al., 1969; Herrold, 1970; IARC Monograph, 1972). MNU is a direct acting carcinogen which breaks down spontaneously in aqueous solutions and does not need to be metabolised to an active molecular species (Swann and Magee, 1968). It interacts with nucleic acids in vitro and in vivo to produce a variety of alkylated products and hence to damage DNA (Cox and Irving, 1976).

Experimental evidence proved that direct acting carcinogens, or those which break down spontaneously to give the ultimate carcinogen, can act directly if instilled into the bladder (Scott and Boyd, 1953; McDonald and Lund, 1954; Santana, 1963; Levi et al., 1969). A single dose of MNU is not a complete carcinogen but it only initiates changes which can be promoted to tumour formation by further doses of MNU or by certain other components such as saccharin or cyclamate (Hicks et al., 1975). However, if 4 doses are used (instilled directly into the bladder), all animals developed tumours of the bladder and of no other organs (Hicks and Wakefield, 1972).

The earliest changes observed in bladder urothelium in experimental carcinogenesis depend on whether the particular chemical carcinogen used is, or is not, cytotoxic as well as carcinogenic. Although, a single noncarcinogenic dose of MNU produced necrosis followed by stripping of much of the urothelium and compensatory benign hyperplasia, a carcinogenic dose produced stripping which was followed by preneoplastic hyperplasia and neoplasia (Hicks and Wakefield, 1972). The toxic damage of the carcinogenic dose was
similar to that of noncarcinogenic dose (Hicks and Wakefield, 1972; Wakefield and Hicks 1973). The earliest subcellular sign of acute damage was oedema, which affected the three layers within a few hours of treatment. The cells became swollen and lost cytoplasmic density. At the same time, there was distension of the extracellular spaces, and the cells separated along their lateral borders. The nuclei showed disruption of their nucleoli and margination followed by clumping of the chromatin. In the worst affected areas, the whole urothelium was desquamated down to the basal lamina, which, however, remained intact.

In early preneoplastic hyperplasia, some of the morphological features associated with benign reversible hyperplasia were often observed. Several investigators have attempted to distinguish the early ultrastructural changes associated with preneoplastic transformation from those that occur with benign, reversible hyperplasia, using transmission and scanning electron microscopy (Hicks and Wakefield, 1972, 1976; Hicks et al., 1974; Hicks, 1976; Hodges et al., 1976; Newman and Hicks, 1977; Hicks and Chowaniec, 1978; Hainau, 1979). The most recent studies conducted by Severs et al. (1982) were aimed at determining the dose response of rat bladder urothelium to a range of different single and fractionated intravesical doses of MNU. They demonstrated that bladder carcinogenesis has many characteristics in common with the classical multistage model derived earlier from studies on mouse epidermis (Berenblum, 1974) rat liver (Peraino et al., 1973) and possibly colon (Reddy et al., 1978) and lung (Witschi and Lock, 1978).

In the present study, fractionated doses of MNU have been used according to the schedule of Hicks and Wakefield (1972) which guarantees induction of bladder tumour in rats. The ultrastructural changes
and luminal surface alterations in the urothelium and the underlying stroma have been examined at various intervals subsequent to the MNU treatment in order to build up a complete picture of the developing bladder cancer. The results obtained have been compared with those of previous workers and have also been used as a framework to analyse the development of certain naturally occurring bladder tumours in man.

OBSERVATIONS

Group I (1 week post MNU)

In bladders that were examined 1 week after the termination of a course of MNU treatment, there were large areas where most of the urothelium had desquamated, leaving only a single layer of cells (Fig. 92). Even these persisting cells seemed to be loosely attached to the basal lamina and hemi-desmosomes were very seldom seen. In many instances, the basal lamina was bare, being completely denuded of epithelial cells (Fig. 93). The persisting cells had a mean area of $119.2 \, \mu m^2$ which is significantly higher than that of the basal cells of normal rat urothelium. The nuclei of the persisting cells were often irregular in shape, but had relatively less of condensed chromatin than that observable in the basal cells of normal urothelium.

The surfaces of the persisting cells, especially the free surface facing the bladder lumen, were drawn into long, slender microvilli containing bundles of microfilaments; these processes were distributed at more or less regular and well spaced intervals. They were of uniform appearance and in some sections measured over 2 $\mu m$ in length. As shown in Fig. 92, the cells were rather loosely organised except for some simple interlocking of adjacent plasma membranes.
Occasionally desmosomes were noticed along apposed microvilli or the lateral cell borders (Fig. 94) and also between the lateral membranes near their luminal surfaces. No typical zonula occludens was ever noticed near the luminal surface of adjoining cells. The scanning electron micrograph shown in Fig. 98 further illustrates the loosely arranged cells with the prominent interspace spanned by long intertwined microvilli of adjoining cells.

A high power electron micrograph of a persisting cell which appears to be almost completely detached from the basal lamina is presented in Fig. 95. The intervening space between the cell and the basal lamina was filled with an amorphous to fibrous substance, the aggregation of which probably formed the basal lamina. Hemidesmosomes were practically absent at the basal border of cells, the majority of which thus seemed to be unattached to the basal lamina. In places, there were indications of more than one basal lamina (Fig. 97). A striking feature of the cells in this urothelium was their well developed granular endoplasmic reticulum with dilated cisternae which were present throughout the cytoplasm and extended to the periphery of the cell. On occasions (Fig. 95 and inset) profiles suggestive of the dilated cisternae being continuous with the cell surface were noticed. The filamentous material in the extracellular space also closely resembled the contents of the dilated cisternae of the endoplasmic reticulum (Figs. 98, 99, inset). The impression gained from examination of a large number of micrographs was that the cisternal contents were directly discharged to the cell surface. The Golgi apparatus was moderately well developed in these cells (Fig. 96). It contained a few parallel cisternae and small spherical vesicles;
there were no large vesicles or condensing vacuoles indicative of secretory activity. The mitochondria appeared normal, were of variable sizes and were distributed throughout the cell.

**Group II (4 weeks post MNU)**

Light microscope preparations of bladder wall examined 4 weeks after the course of MNU treatments showed that the urothelium varied in thickness from 3 to 7 layers (Fig. 100). The stratification of cells however was not clear cut because of focal variations in cell sizes and shapes, but the urothelium as a whole, seemed compact. The superficially placed cells were also variable in size and shape and in places, the cells appeared cuboidal and larger than the cells of the underlying layers of the hyperplastic urothelium. In other areas, the cells were uniformly small and rounded. Focal areas of necrosis were also observed.

Transmission electron micrographs such as that shown in Fig. 101 confirmed the presence in some areas of large superficial cells facing the lumen of the urothelium. The intercellular cisternal space between these cells was very narrow and the cell-to-cell interdigitation (Fig. 104) was as in normal urothelium. Owing to the large area of the cytoplasm and the small size of the nuclei, the nucleo-cytoplasmic ratio of these cells was low at about 1:5. Representative micrographs of the apical cytoplasm of the superficial cells in question are presented in Figs. 102-105. A constant feature in all instances was the presence of many vesicles of different shapes and sizes adjacent to the luminal plasma membrane. They were neither as numerous nor predominantly fusiform as in the superficial cells of
the normal rat urothelium (Fig. 15). Typical fusiform vesicles with asymmetrical unit membrane were noticeable (Figs. 102, 105, 108) sometimes more in one cell than the adjoining one, but examination of a large number of cells showed that there were also many spherical vesicles limited by symmetrical unit membrane. The lateral membranes of adjoining cells were joined together by junctional complexes (Fig. 104) which, at first sight, appeared to be as in normal urothelia. Well formed tight junctions between the lateral membranes near the luminal surface, as in normal urothelia (Fig. 5), however, were hardly ever met with; instead the junctions appeared to be attenuated, or defective, or of zonula adherens type (Figs. 105, 108). Another feature of these cells was the presence of polymorphic bodies (Fig. 103) as in the superficial cell of the normal urothelium. But unlike in the superficial cell of normal urothelium, the mitochondria were seldom aggregated; they were usually scattered throughout the cytoplasm but appeared normal in their ultramorphology. Some of the superficial cells (for example, Fig. 103) contained more microfilaments than are usual in the normal urothelial cells, and, instead of being randomly orientated, the filaments exhibited a tendency to form bundles.

The transmission electron micrographs shown in Figs. 102-105 draw attention to the variable nature of the free luminal surface of the superficial cells described above. It does not possess the scalloped profile so characteristic of the luminal surface of the normal rat bladder urothelium (Fig. 15). For the most part, the surface appeared to be gently folded and the folds were by no means regular. It is such regions that most probably account for the flat, pavement-like surface with irregular blebs in scanning electron micrographs.
(Fig. 109). In other regions, sometimes adjoining that just described, transmission electron micrographs showed short, closely spaced, microvilli as in Fig. 104. A scanning electron micrograph of what is believed to be such a surface is presented in Fig. 111. In transmission micrographs, the luminal membrane appeared symmetrical in most parts and asymmetrical in others. The former structure was more apparent in the region of the folds and the microvilli whereas the latter was often associated with the more straight regions of the surface or in the regions in-between microvilli.

Several layers of intermediate cells were present in the hyperplastic regions of the urothelium and those immediately adjacent to the superficial cells described above often showed vesicles in the apical cytoplasm (Fig. 101) as in the superficial cells. Some of them also contained polymorphic bodies. These features were not visible in the deeper layers where all cells including those in the basal layer were undifferentiated and morphologically alike. The nuclei of the intermediate layers of cells as well as those of the basal layer were somewhat different in appearance from that observed in normal urothelium. The differences were mainly that, much more of the chromatin was in a diffuse state than in normal urothelium and the nucleoli were more prominent. Both in the basal and intermediate cells, the cells sporadically displayed increased bundles of microfilaments (Fig. 106).

Other focal areas of the hyperplastic urothelium were made up of smaller rounded cells with strikingly rounded nuclei and practically all their chromatin in the diffuse state (Fig. 107). In transmission electron micrographs, the luminal surface of such cells in the superficial layer showed a regular, wavy contour with crests occasionally
raised into microvilli; what is believed to be the appearance of such a surface in the scanning electron microscope is presented in Fig. 110. Apart from the features already mentioned, these cells contained numerous bundles of microfilaments in the cytoplasm and increased numbers of desmosomes with which some of the filament bundles were obviously continuous (Fig. 107). The cytoplasm was also unusually rich in free polysomes. The mitochondria of these cells seemed normal but some of them were unusually long (up to about 4.5 μm) with closely spaced transverse cristae.

Some unusual observations in the urothelium of the rats examined 4 weeks after the course of MNU treatment were in the region of the urothelial-stromal interface (Fig. 106). Although not many instances were met with, the basal lamina below the basal cells appeared to be interrupted in places. In addition, there were also instances where blood capillaries seemed to be pushing into the urothelium. Such inpushings of capillaries occurred not only in regions where there were apparent breaks in, or rarefied appearance of, the basal lamina but also in regions where no such interruptions were visible. In locations of such close association between the capillary vessels and the urothelium, there was also a dearth of collagen fibres.

**Group III (12 weeks post MNU)**

Twelve weeks after the termination of the intravesicular MNU instillations, the bladder urothelium continued to be hyperplastic with up to eight layers of cells. In certain regions, the hyperplastic urothelium projected into the lumen of the bladder in the form of simple papillary processes or pappilomas. The papillomas were 5 to 8 cell layers thick and the superficially placed cells
appeared at first sight to be similar to those in the deeper layers. A central core of blood capillaries and supporting stroma were present in the papillary projections (Fig. 112). In focal areas of the hyperplastic urothelium of the papillary processes, there were present cells with numerous bundles of cytoplasmic filaments. Areas of necrosis were still evident at different foci in the urothelium.

The multifocal formation of papillary projections was the most significant feature of this urothelium. The transitional cells were less cuboidal in shape than in the previous group and had very irregular nuclei which also were variable in size. The cells and nuclei of the peripheral layers were slightly larger than those in the deeper layers of the hyperplastic urothelium and the nucleoli of these cells tended to be more prominent. There was a slight gradation in the proportion of chromatin in the diffuse state; the nuclei of the superficially placed cells and the immediately adjoining intermediate cells had a greater proportion of their chromatin in a diffuse state than those of the cell layers below. The intercellular cisternal space between cells at all levels continued to be narrow with apparently normal, cell-to-cell interdigitation (Figs. 113, 115).

The organelle content of the transitional cells was generally low, although many scattered profiles of rough endoplasmic reticulum and moderately developed Golgi complexes with vacuolar entities were evident in cells at different levels. In high magnification pictures, the superficial cells showed the presence of many free vesicles in the apical cytoplasm. Some of these were either fusiform or near fusiform in shape and were bounded by asymmetric unit membrane (Figs. 114, 116, 118, 120) while others were rounded vesicles with a symmetric limiting membrane. Patches of asymmetrical membranes were probably
present, also as in the previous group, at the luminal surface (Fig. 119). The luminal surface as a whole seemed mainly microvillous. The microvilli in this instance were separate finger-like processes, closely but irregularly spaced, and of variable sizes measuring up to 0.5 \( \mu \text{m} \) in height. Some of them were also branched (Figs. 114, 117, 119, 120, 123). Additionally, the urinary face of the urothelium was covered with a layer of electron-dense material, the cell coat or glycocalyx (Fig. 119). As a result it was difficult to discern the ultrastructure of the luminal plasma membrane except in favourable instances at very high resolution. In such preparations (Fig. 118), the microvilli appeared to be limited by symmetrical membrane.

Junctional complexes were evident along the lateral borders of the superficial cells (Figs. 114, 118, 120), but as far as could be judged from electron micrographs of thin sections, it seemed unlikely that normal tight junctions existed near their luminal surfaces. The majority of junctions at this location had the appearance of zonula adherens except perhaps at the very edge where an attenuated tight junction may be present (Fig. 118). The exact nature of this junctional formation remains to be clarified by further studies using freeze-fracture techniques.

The surface of a papillary process in the scanning electron microscope is shown in Fig. 122. It was clear that the superficial layer was not formed by large, flattened cells. Instead, the cells were more rounded like cobblestones and were variable in size. In higher power (Fig. 123), the surfaces of superficial cells were seen to display separate, finger-like processes, a few of which were branched. The presence of the glycocalyx was not visualized in the scanning micrographs.
as readily as in the transmission pictures.

The intermediate cells present in several layers were more or less similar in size and ultramorphology (Fig. 115). Their nuclear envelopes were indented and, in the cells of lower layers, a margined layer of condensed chromatin was more obvious than in the upper layers. However, this was a not very far from consistent feature. In the penultimate layer of intermediate cells, only a few contained free vesicles in the apical cytoplasm and when present, they were almost all spherical. The lower layer of intermediate cells, passed almost imperceptibly into the basal layer. Many more of the basal cells possessed micro-filament bundles than in the upper layers.

In focal areas of the hyperplastic urothelium, a different kind of cell was met with and they were found mostly in the basal and intermediate levels. They were not very different in size from the lower layers of cells already described and their nuclei were also indented. Their most distinguishing feature was the presence of numerous filament bundles and increased numbers of desmosomes (Figs. 121, 124). Some of the filament bundles appeared to be continuous with desmosomes. The cells were also richly endowed with free polysomes amidst which profiles of rough endoplasmic reticulum could be made out. In their cytoplasmic features particularly, these cells resembled the cells with rounded nuclei that were observed in focal areas of the urothelium 4 weeks after MNU instillations.

The urothelial-stromal interface in this urothelium also showed unusual juxtaposition of cell types. One of these frequently observed was the intimate contact between blood capillary and the basal cells (Figs. 124-126). The basal laminae at these sites appeared intact in some instances (Fig. 125, 126) and not so in others (Fig. 124).
In micrographs such as presented in Fig. 127, it looked as though a capillary was located within the urothelium. Also, visible in the stroma in Fig. 126 are capillaries in intimate contact with the urothelium. Lymphocytes and polymorphonuclear leukocytes were also encountered interposed between urothelial cells at all levels.

Group IV (24 weeks post MNU)

Twenty-four weeks after the termination of the course of MNU instillation, the entire urinary face of the bladder was in the form of long papillomas and nodular aggregates. The long papillomas and their branches projecting into the bladder lumen consisted of transitional cells arranged around a central core of blood vessels and supporting stroma. The papillary and nodular hyperplasia was four to eight cell layers thick and was composed of relatively small cells which were more or less uniform in size and morphology from the basal layer to the surface (Fig. 128). An interesting feature in the urothelium was the presence of small luminae in the superficial and intermediate cells. Keratinised areas of squamous metaplasia or keratin plaques were not observed.

Low power transmission electron micrographs (Figs. 129, 132, 134, 137) show the general appearance of the urothelium with somewhat wide intercellular spaces. These spaces contained long microvilli-like processes of adjoining cell borders interdigitating with each other. Desmosomes were frequently visible at all levels in the hyperplastic urothelium. Some of the most striking features of the urothelium were at the luminal surface which possessed numerous closely spaced microvilli of varying dimensions covered with a thick electron dense cell coat. More will be said of these later. The intercellular
junctions at the luminal surface hardly ever appeared to be normal (Fig. 133). The impression gained from examination of a number of micrographs was that only an adherens junction, probably a zonula adherens, held the adjoining cells together at the luminal surface.

Representative micrographs to show the ultrastructure of superficial cells are presented in Figs. 129, 130, 141). There were only few rounded vesicles in the apical cytoplasm adjoining the luminal plasma membrane. The mitochondria were scattered throughout the cytoplasm, unlike in normal cells where they tended to localize the apical region. Also, several of the mitochondria appeared to be abnormal and bloated. There were no polymorphic bodies of the kind described in normal urothelial cells, although other uniformly dense bodies were present. One organelle which was relatively prominent in these cells was the rough endoplasmic reticulum which seemed to be more extensive than in the cells of normal urothelium; profiles of this endomembrane system were present throughout the cytoplasm. Several small, but independent, Golgi complexes were present in the cell and areas of interaction between the endoplasmic reticulum and the Golgi complex were often met with. The cytoplasmic matrix contained an abundance of free ribosomes and polysomes.

The ultrastructure of the cells situated deeper in the urothelium was similar to that described above. Several profiles of rough endoplasmic reticulum and a large number of ribosomes and especially polysomes were clearly visible in the intermediate and basal layers of cells (Figs. 131, 132, 134, 135, 137). The Golgi complexes in these cells, as in the superficial cells, were not consistently confined to any particular location.

The mitochondria in the cells of all layers were distended and
appeared abnormal or deformed with incomplete cristae. Some of the mitochondria did not show any internal organisation and there were a few which were obviously undergoing lysis with the formation of myelin-like figures. The micrographs presented show that in general, microfilament bundles were not prominent in the upper layers of urothelial cells of this group. Cells containing such bundles were more likely to be found in the lower layers, including the basal layer (Figs. 131, 135, 137). The basal lamina was generally uninterrupted and its ultrastructure was as in normal urothelium. In rare instances where breaks were seen, basal cell pseudopods projected through the gap into the stroma (Fig. 140).

The nuclei of all the urothelial cells in this group were more or less similar in their ultramorphology, and contained masses of condensed chromatin distributed throughout the nucleus and especially along the nuclear envelope. The nucleoli in all the cell layers were compact with their fibrilli-granular constituents organised in the form of a clear nucleolonemal network.

As stated earlier, some of the most striking features of the urothelium, examined 24 weeks after a fractionated course of MNU treatments, were in the upper cell layers, especially that lining the bladder lumen. The occurrence of characteristically pleomorphic microvilli projecting into the lumen from the luminal layer of cells was already mentioned. In thin sections used for transmission electron microscopy, it was not easy to observe the variability of the sizes of the microvilli but on occasions, it was possible to note the existence of some measuring nearly 3 μm in length (Fig. 136). Likewise, their diameters also varied from nearly 0.13 μm to about 0.3 μm.
Several microvilli were also branched. A very thick layer of surface coat material covered the entire luminal surface and the microvilli; a thickness of up to about 90 nm has been found in some sections, and it was extremely electron dense following the routine staining with uranium and lead (Figs. 130, 133, 136, 138, 139, 141). The cell coat, in other words, was strikingly obvious in ordinary preparations made without any special fixing and/or staining procedures. The huge amount of glycocalyx made it very difficult to visualize the ultrastructure of the luminal plasma membrane but in favourable instances such as transverse sections of microvilli (Fig. 138) the limiting membrane was found to be symmetrical.

Another feature unique to the papilloma of this group was the occurrence of intracytoplasmic luminae. These formations were essentially new or secondary luminal surfaces appearing in the superficial cells or in the cells of the immediately adjacent lower layer (Fig. 141). The intracytoplasmic luminal surfaces possessed pleomorphic microvilli covered with glycocalyx, as in the main luminal surface. Associated with this phenomenon was another similar manifestation. This consisted of an extension of the luminal surface characteristics of the superficial cells to their lateral and basal surfaces. A matching manifestation appeared to have developed precociously at the apical surface of some cells of the lower layer to give rise to what may be called intercellular luminae (Figs. 136, 141). Examination of serial sections indicated that some of the intracytoplasmic luminae were continuous with the principal luminal surface via the intercellular luminae.

Examination of the urinary face of the bladder by scanning electron microscope showed numerous papillomas protruding into the lumen (Fig.
Higher power pictures revealed the presence of large cave-like or tunnel-like openings on the luminal surface of the papillomas and nodular aggregates (Fig. 143). In all probability they represented the openings of the secondary luminae which, as explained above, formed in the hyperplastic urothelium. Also visible at the surface were some erythrocytes due to haemorrhage because the papillary tumours were probably fragile and very vascular. In high magnification scanning electron micrographs, the profuse microvilli of the luminal surface were visible but their clarity was poor (Fig. 144), presumably because they were embedded, so to speak, in the thick glycocalyx.

**DISCUSSION**

In this group of experiments with MNU, the rats were given 4 bi-weekly doses of 1.5 mg, each directly into the urinary bladder. Such multiple, intravesicular doses given over a period of 6 weeks have been shown previously by Hicks and Wakefield (1972) to produce bladder tumours in all treated animals. MNU which breaks down spontaneously to an ultimate carcinogen appears to be the most potent direct acting carcinogen so far known for the urinary bladder and the total dose of 6 mg administered as above was used to ensure a 100% tumour incidence. The half-life of MNU is very short, being about 1.2 hours at pH 7 (Druckery *et al.*, 1967), and the actual exposure of the urothelium to this carcinogen required to produce tumours also seems to be brief. Although the 4 doses were spread over a period of 6 weeks, the actual duration of contact with the urothelium was probably less than 2 hours in total, because as was pointed out earlier by Hicks and Wakefield (1972), the animals expelled
the MNU by micturition within an average of about 20 minutes of instillation. The reactivity of MNU is thus exceptional since it had been generally assumed (see Clayson and Cooper, 1970) that a period of continuous administration of a chemical carcinogen is necessary for the induction of bladder tumours.

Like many other bladder carcinogen, MNU is also cytotoxic for the urothelium and at first causes toxic damage and/or cell death. The initial sequence of events in rats after a single dose of 2 mg MNU administered intravesicularly has been described before (Wakefield and Hicks, 1973). The earliest subcellular sign of damage was oedema, affecting cells in all the layers within a few hours of treatment. The urothelial cells became swollen and many lost cytoplasmic density. In the nuclei, the chromatin became clumped and marginated and nucleoli were disrupted. Within about 12 hours, the cells started to separate and the intercellular spaces became grossly distended. By this time most superficial and many intermediate cells were necrotic and there was extensive desquamation within 24 hours of dosing. In large areas of the bladder where the damage was most severe, the epithelium was stripped completely by about 5 days to leave a bare but usually intact basal lamina.

As a result of extensive desquamation, the smaller, more rounded intermediate and basal cells came to the luminal surface in many regions and these surviving cells exhibited several ultrastructural alterations. According to the above mentioned workers (Wakefield & Hicks, 1973), within 2 days of the treatment, lipid droplets appeared in the cytoplasm of the cells, especially the superficial cells, and there were also many aberrant mitochondria, which indicated impairment of tissue respiration.
These cells also were probably shed subsequently because, within 2 weeks after the single, noncancerogenic dose of MNU, the urothelium regenerated rapidly and was composed of many layers of immature cells. The cells of the hyperplastic (eight to ten layers) urothelium were morphologically similar and showed no differentiation between basal and superficial layers. A striking feature of the hyperplastic urothelium during the second and third week after the MNU treatment was the increased number of desmosomes, cytoplasmic filaments, and aggregates of such filaments forming what are usually called, tonofibrils in all cell layers.

From the data provided by earlier workers (Hicks and Wakefield, 1972; Wakefield and Hicks, 1973), it may be safely assumed that when the second dose of MNU was applied in the present experiment, the urothelium was in a hyperplastic or regenerated state, although focal areas of necrosis might well have been present. One may also be reasonably certain that there was no differentiation of the cells within the hyperplastic urothelium (that is within 2 weeks of a single dose of MNU). There is not much information in the literature on the consequence of successive intravesicular doses of MNU at fortnightly intervals. But, after each dose, one might expect the toxic effects of the chemical on the urothelium to be like those after the first dose, leading to focal areas of necrosis and desquamation, and regeneration leading to hyperplasia in other areas. The sequence of events, leading to the regeneration and repair was probably like that following any injury or acute insult to the bladder urothelium (see review by Clayson, 1975). It would seem likely that such cycles of necrosis and regeneration resulting in hyperplasia in some areas were repeated after the third and fourth doses of MNU.
applied in the present study.

A week after the final dose of the fractionated treatment, the urothelium was totally stripped in many areas, and in other areas there was only a single layer of cells, not unlike the state of the urothelium described by Hicks and Wakefield (1972) five days after a single dose of MNU, or the urothelium described by Koss (1967) and Koss and Lavin (1971), after a single dose of cyclophosphamide in other strains of rat. The population of persisting cells in the present instance, however, was of a different nature. They were generally larger than the basal cells of the normal rat urothelium. Their nuclear morphology was also different, with more chromatin in a diffuse state. In many of the cells, the granular endoplasmic reticulum was not only extensive but also distended with an amorphous to fibrous substance which was not unlike the substance described in the literature as forming collagen or tropocollagen. Such a substance was also visible at the surface of some cells, although no typical banded collagen fibrils were discernible. All in all, many of the cells in the persisting layer of the urothelium, 1 week after four repeated doses of MNU, have a morphological resemblance to fibroblasts. If these cells are indeed of the nature of fibroblasts the tropocollagen produced must be shed into the bladder lumen and voided into the urine. The nature of the substance produced in the rough endoplasmic reticulum and apparently secreted into the cell surface requires to be confirmed for example by treatment of the cells by collagenase as described by other workers (Goel and Jacob, 1976).

The occurrence of cells resembling fibroblasts has not hitherto been reported in the urothelial cells subjected to toxic or other damage. There are, however, reports of urothelial cells, not killed
by various treatments, exhibiting ultrastructural alterations (cf. Hicks and Chowaniec, 1978). For instance 2 days after treatment with MNU, methylmethanesulfonate (MMS), ethylmethanesulfonate (EMS) or the sulfonamide ENS, irregular lipid droplets have been reported to form within the cisternae of the rough endoplasmic system (Wakefield and Hicks, 1973). Another alteration of urothelial cells is that described by Hicks (1969/1970) and Wakefield and Hicks (1973); in urothelium regenerating in response to inflicted damage, the rapidly dividing urothelial cells behaved like phagocytes. In the present instance, one cannot be certain if the fibroblast-like cells are derived from stem cells of the urothelium. It may be relevant to note that although the main part of the urinary bladder originates from the cloacal endoderm, the trigone is formed from the mesodermal epithelium of the Wolfian duct. On the other hand, there is no knowing if the fibroblast-like cells in question arose from cells migrating from the supporting mesenchyme in order to populate a practically bare urothelium. There is evidence in the literature (Constantinides, 1974) to suggest that new fibroblasts may arise not only from pre-existing fibroblasts by mitosis but also from immigrant blood cells (monocytes?). Infiltration of the urothelium by elements of the subepithelial region is well known and can occur in a variety of circumstances especially during necrosis and haemorrhage following acute damage. For a long time, it was believed that only fibroblasts were capable of synthesis of collagen and this function was essential to the definition of the fibroblast. With the advent of electron microscopy, it has been found that cells with the ultrastructural character of smooth muscle are capable of laying down collagen. More unexpected has been the finding that certain, ectoderm-derived cells may secrete
fibrillar collagen (Gould and Battifora, 1976).

Another observation in this urothelium was the occurrence of more than one basal lamina or unclarified basal lamina of unusual width. Mature epithelia have a characteristic extracellular layer subjacent to their basal surfaces, which is referred to in electron microscope preparations as the basal lamina. Basal lamina is synonymous with the names, basement membrane, basement lamina, basal lamella or basement lamella. The so-called basement membrane as seen in light microscope preparations consists in the electron microscope of lamina lucida and lamina densa together with their immediately subjacent connective tissue fibres. The basal lamina appears under developing epithelia at various times during development and is generally regarded as a product of the epithelial cells (Pierce et al., 1964, 1967; Smith, 1972; Vracko, 1974). The occurrence of multiple lamina has been attributed by many workers to injury which caused the epithelium to synthesize additional laminae (Pierce et al., 1969; Smith, 1972; Koss, 1977). In all probability, the instances of multiple lamina in the one week post MNU urothelium are the marks left behind by successive generations of cells during cell proliferation at the time of recovery from each dose of the chemical.

Four weeks after the termination of the multiple doses of MNU, the urothelium was clearly hyperplastic with up to seven layers, although focal areas of necrosis were still visible. The proliferative response was similar to that observed after both non-carcinogenic and carcinogenic doses of MNU (Hicks and Wakefield, 1972; Wakefield and Hicks, 1973), or after a single dose of cyclophosphamide (Koss, 1967), or other acute damage to the urothelium (Hodges, 1979). In certain areas the hyperplastic tissue was composed of small rounded
cells whereas, in other areas, there were indications of cytodifferentiation in the relatively larger, cuboidal cells of the superficial layer and the penultimate layer of intermediate cells. The indication of cytodifferentiation was the presence, in the apical cytoplasm of these cells, of fusiform vesicles bounded by asymmetrical membrane. The cells of the hyperplastic urothelium under discussion showed little resemblance to the cells observed at 1 week after the multiple doses of MNU. Whatever the origin and nature of the latter (discussed earlier), they were subsequently lost by desquamation and, between weeks 2 and 4 post MNU, the urothelium was reconstituted with a new population of smaller cells. These cells were derived, presumably, from stem cells that survived the MNU treatments with perhaps delayed but otherwise unimpaired proliferative capacity. Such a property of stem cells has been repeatedly pointed out in experimental animals (see Clayson, 1975) and in human bladder urothelium (Jacob et al., 1982).

As already explained, the multilayered urothelium formed 4 weeks after the termination of the carcinogenic dose of MNU contained cells with some overt signs of cytodifferentiation although there was no organised pattern of differentiation from the base to the surface. Urothelium containing differentiating cuboidal cells has been found in both benign, reversible hyperplasia (where normal differentiation will result if there is no further damaging stimulus), as well as in hyperplasia leading to papillomas and transitional cell tumours (cf. Koss, 1977; Hicks and Chowaniec, 1978). In addition, there were present in other areas of the hyperplastic urothelium under discussion, nests of more rounded cells with rounded nuclei containing a large number of dense, laterally aggregated bundles of filaments in the cytoplasm resembling the filament aggregates referred to in the
literature as tonofibrils. The development of small islands of squamous metaplasia has been previously reported in both MNU- and DBN-transformed urothelia (Hicks and Wakefield, 1972; 1976): at the urinary face of some of those bladders, squamous metaplasia was associated with the formation of keratin plaques. A similar picture of numerous tonofibrils and cornification was observed by Hicks (1968), Ghidoni and Campbell (1969) and Hicks et al. (1976) in benign squamous metaplasia in the hyperplastic urothelium of rats induced by deficiency of vitamin A in the diet. The transitional epithelial cells of the normal bladder contain 8-10 nm thick cytoplasmic filaments, which at the present time are usually referred to as intermediate filaments and which many authors believe to be structural precursors of keratin. In normal bladder the filaments are sparse and randomly oriented. In the cortical region of the luminal layer of cells, they run parallel to the long axis of the cell and are associated with the desmosome type of junctions of the junctional complex forming the so-called "terminal web" of early histological literature. The hyperplasia and cornification of the bladder urothelium of rats deficient in vitamin A suggests that the epithelium lining the bladder, like that of the mouth, oesophagus and vagina is a keratin-synthesizing tissue that does not normally cornify except in response to some stress. It may be of interest to mention here that cytokeratin filaments are now known from immuno-fluorescence studies to be present in a wide variety of the well known epithelia (Sun et al., 1979).

Greatly increased numbers of tonofibrils as well as desmosomes have been found in hyperplastic urothelium of experimental animals recovering from mechanical damage and carcinogenic and non-carcinogenic treatments (Hicks and Wakefield, 1972, 1976; Wakefield and Hicks, 1973). The bladder urothelium is capable of squamous metaplastic changes
under a variety of conditions and it has been suggested (Hicks, 1976; Hicks and Chowaniec, 1978) that this change is an alternative form of differentiation involving the hyperactivity of an existing metabolic pathway at the expense of others rather than a change in differentiation involving alteration to the genome. According to Hicks and Chowaniec (1978), the ease with which normal transitional epithelium can transform into an epidermal type of tissue suggests that the latter may represent a lower state of entropy to which the tissue will regress unless actively maintained in the state of normal transitional differentiation by interplay of epithelial and mesenchymal factors. Hence squamous metaplasia in a hyperplastic urothelium is not regarded as automatically indicative of neoplasia or as an integral part of the neoplastic process. A contrary view has been expressed by Koss (1977). In view of the widespread manifestation of squamous metaplasia early in experimental chemical carcinogenesis and its later invasive nature, Koss subscribes to the view that this represents a genomic alteration causing metaplastic transformation and concomitant capacity for uncontrolled cell proliferation. This author presumably implies that the squamous metaplasia arising as a result of treatment by chemical carcinogens is the result of genetic mutation caused by interaction of carcinogen with urothelial cell DNA and must be distinguished from the reversible squamous changes in hyperplastic urothelium induced by deficiency of vitamin A. The latter appear to be more akin to the phenomenon termed modulation rather than metaplasia (cf. Ede, 1978).

The MNU regimen adopted in this study was such that all the animals treated were almost guaranteed to develop papillomas and transitional cell tumours within 4 to 6 weeks of the termination of the treatment.
In view of this, the hyperplastic urothelium examined 4 weeks after the treatment may be considered to be preneoplastic. Yet, there were hardly any incontrovertible structural features in the urothelium to distinguish it from benign hyperplasia. The variable appearance of the luminal surfaces in the scanning electron micrographs ranging from fairly smooth to occasional ridges or from small blebs to globular projections were also like those described by several workers during benign hyperplasia or during recovery from other injuries.

On the other hand, it would seem that more significant changes take place in the interface between the urothelium and the lamina propria, and in the lamina propria itself. As explained elsewhere, there were occasional breaks in the lamina densa or rarefied regions of the basal lamina of the urothelium. In many such instances there was also a close association of capillary vessels with indistinct basal lamina. These changes including the apparent approach of the capillaries to the urothelium, which occurred early in the course of the experimental carcinogenesis, assume great importance when considered in the light of changes observed at a later period - that is, in specimens examined 12 weeks after the termination of the MNU treatment.

Transitional cell papillomas were clearly formed in the hyperplastic urothelium of bladders 12 weeks after the course of MNU. The constituent cells were generally undifferentiated cells with a low content of organelles. Their nuclei were irregular and variable in shape but with prominent nucleoli. The cells may be described as pleomorphic and surprisingly, there were still visible asymmetric membranes in the form of free vesicles in some of the superficially placed cells. Unusual for urothelial cells, however, was the
prominence of the rough endoplasmic reticulum in individual cells at all levels in this hyperplastic urothelium. A distinct population of cells in scattered groups was also encountered in this urothelium which corresponds to the development of squamous metaplasia. But unlike in the experiments of Hicks and Wakefield (1972), no intra-urothelial pearls or keratin plaques were visible in association with the squamous metaplasia in the material examined in the present study.

The urothelium under discussion with pleomorphic cells must be regarded as preneoplastic, and the hyperplasia must be regarded as irreversible. Apart from the pleomorphism of the cells and their varying ultramorphology, there were other notable features associated with this phase of developing tumours. These features were at the urinary surface as well as at the epithelial-mesenchymal interface and in the lamina propria.

At 12 weeks after the MNU treatment, the formation of finger-like microvilli at the luminal surface generally, with occasional branched forms, and the simultaneous presence of a glycocalyx are very significant developments. The microvilli are separate finger-like processes which are longer than microvilli observed on some surface regions of the urothelium at 4 weeks after the MNU treatment. These surface differentiations, i.e. microvilli, differ in height from the surface differentiations referred to as ridges in some publications but they have many similarities and share some important properties, as discussed recently by Breiter-Hahn et al. (1979). In view of their similarities, it may not often be easy to distinguish them on the basis of separate transmission electron microscope or scanning microscope studies. They are probably best made out
in combined studies. This difficulty may probably explain the considerable amount of confusion that exists in the electron microscope literature relating to the presence or absence of one or the other surface feature in normal and abnormal urothelial surfaces.

At the same time as the microvilli are formed in plenty at the luminal surface of the urothelium, i.e. 12 weeks after MNU instillation, an unmistakable glycocalyx makes its appearance for the first time in this series of urothelia. It is important to stress the fact that the glycocalyx becomes visible in the preparations stained only by routine techniques as used for this entire study. It is now generally accepted that the outer surface of the plasma membrane of most animal cells is covered by a glycoprotein layer forming the glycocalyx or cell coat, and that this can be demonstrated if sensitive enough methods are used. A delicate layer of glycocalyx is made evident with special stains on the luminal surface or normal bladder urothelium (Koss, 1977). However, in many cell types stained by routine techniques, the glycocalyx is not usually seen even in the electron microscope, and this is also the case in normal rat urothelium and in the hyperplastic urothelium at 4 weeks after the carcinogenic treatment. It is likely that a glycocalyx is actually present in these urothelia and can be demonstrated by staining techniques specific for carbohydrate residues of the glycoproteins (see review by Luft, 1976). Yet, at 12 weeks after the carcinogenic treatment, the luminal surface shows a readily visible filamentous/beaded glycoccalyx without any special staining techniques. This in itself is not unique because in many other instances, described in the literature, for example, (Bennett and Leblond, 1976) in the apical region of the surface columnar cells from the ascending colon
of mouse, the cell surface coat is visible without any special staining techniques. Also, a thicker than usual surface coat is not invariably found in cancer cells (cf. Sandford et al., 1976). But the significance in the present context is that at 12 weeks post carcinogenic treatment, a glycocalyx with a substructure is visible without any special staining for the first time in the series of bladder urothelia examined, and this development must be judged in the given setting and context of developing neoplasia in a particular tissue system.

The intercellular sites of biosynthesis of the glycoproteins of the plasma membrane is now fairly well understood and it is probable that the rounded vesicles in the apical cytoplasm of the luminal layer of cells are the carriers of the glycocalyx (as components of their bounding membrane as in other cells studied, Bennett and Leblond, 1976). The visibility of the glycocalyx in the urothelium under discussion may be due either to a more profuse production of the substance than before or to changes in the structure and quality of the glycoprotein molecules. The presence of many profiles of rough endoplasmic reticulum and Golgi apparatus in the constituent cells of this urothelium indicate the former possibility, while biochemical data would be required to establish qualitative changes. According to our present knowledge, the carbohydrate components of glycoproteins are involved in determining cell adhesiveness, cell deformability, cell surface charge, transport processes and antigenic properties. Any of these functional characteristics may be affected by the observed accumulation or broadening of the glycocalyx. It must be remembered too that considerable evidence has accumulated in the recent past implicating the cell surface as the site of important changes consequent on neoplastic transformation. The time of materialization of the cell coat
and its ultrastructural diversity may well reflect the molecular variability of the glycoproteins and glycolipids (Luft, 1976).
The addition of glycoproteins to the plasma membrane has different significance in different cell types and in some cells at different periods of development. In the context of the present discussion, the relevant information from literature that may be cited are those pertaining to transformed cells. To mention a few, Warren et al. (1972) have described an increase in fucose-containing glycoproteins on the surface membranes of chick embryo fibroblasts transformed with a virus and Mallucci et al. (1972) have provided data to show that transformed cells have an increase in the rate of cell coat synthesis. Likewise, Martinez-Palomo and co-workers (1969) have observed a widening of the glycocalyx in hamster cells transformed by SV40 viruses.

As so many workers have reported, microvilli develop in benign or reversible hyperplasia of the bladder urothelium as well as in certain other normal circumstances and consequently the view is gaining increasing acceptance that the presence on the urinary face of microvilli in itself cannot be regarded as a specific indicator of neoplastic change (cf. Hicks and Wakefield, 1976; Davies and Hunt, 1981). But the present evidence strongly suggests that in the rat model when the microvilli of a hyperplastic urothelium are accompanied by the materialization of, not a simple glycocalyx, but one with a substructure, following routine staining procedures, then the urothelium is very likely to be neoplastic. An ultrastructurally simple and thin glycocalyx may occur in some benign hyperplastic urothelium such as that for instance induced by a single dose of MNU but never in normal, untreated, adult rat urothelium after routine
staining (Hicks, 1976; Newman and Hicks, 1977; the present study).
The present observations reinforce the view that such ultrastructural
dparameters can provide indications of neoplastic transformation and
that urothelia with such multiple features will develop into trans-
itional cell tumours (Hicks and Chowaniec, 1978).

At 12 weeks after the carcinogenic treatment several changes
were noticeable in the lamina propria and in the interface between
this and the urothelium. On these, the breaks in the basal lamina
which were observed even during the early regenerative phase do not
seem to be particularly significant, as explained earlier. Among the
various ultrastructural disturbances evident in the lamina propria
of the urothelium under discussion, the one that appears to be a
gradually developing pattern is the increasing association between
urothelium and blood capillaries. Further to the intimate associa-
tion between the two, noted in the 4 weeks post MNU bladder, some
capillaries in the 12 weeks post MNU sample seem to have actually
entered the urothelium. An early proliferation of capillaries below
and into neoplastic urothelium and into other tumours has been des-
cribed in the past (for example: Algire and Chalkley, 1945; Wood,
1958; Warren and Shubik, 1966; Cavallo et al., 1972; Hicks and
Chowaniec, 1978; Severs et al., 1982). Suggestions have also been
made that stimulation of capillary growth may be mediated by a
diffusible humoral factor - the tumour angiogenesis factor - produced
by tumour cells and which is mitogenic for capillary endothelial
cells (Algire and Chalkley, 1945; Warren and Shubik, 1966; Fulkman
et al., 1971; Cavallo et al., 1972); the tumour angiogenesis factors
(TAF) are able to diffuse through a millipore filter and stimulate
capillary growth. TAF have been extracted and purified and shown to
induce DNA synthesis in capillary endothelial cells as far away as
3-5 mm from the site of injection (Folkman, 1974). Hope has been expressed that blocking the synthesis of TAF and/or inhibiting it after its secretion by tumour cells may prove to be a potent form of cancer chemotherapy (Folkman, 1974; Oppenheimer, 1983). In keeping with these ideas, if the ability to produce TAF is an early change induced simultaneously with an ability for uncontrolled cell division as a result of the interaction of the carcinogen with the DNA, then an observation made in the urothelium at 4 weeks after MNU assume great importance. The observation, it may be recalled, was a developing intimate contact between blood capillaries and urothelium as though a signal from neoplastically transformed cells has triggered capillary proliferation. One might, therefore, venture a suggestion that perhaps, a definitive, morphological indication of neoplastic transformation occurring earlier than those mentioned above in the urothelium may be found in the sub-epithelium. Added support for this suggestion comes from the fact that such a capillary proliferation has not been reported so far in experimental bladders of animals subjected to various kinds of injuries and toxic treatments, even though capillary regeneration and proliferation are known to occur (cf. Folkman, 1974) in other tissues during wound healing and in response to some inflammatory agents.

At 24 weeks after the MNU treatment, the papillary tumour formed are mainly transitional cell tumours basically similar to that produced by prolonged administration of DBN and BBN (Druckery et al., 1962; Ito et al., 1969; Bertram and Craig, 1970; Okajima et al., 1971). Apart from the transitional cell tumours, there were also areas of developing squamous metaplasia in the intermediate and lower layers of the hyperplastic urothelium but no keratin pearls were seen. There
would be little problem in identifying cornification if it was present but no such areas or plaques of keratin on the free surface were seen in the present study. These formations have been described before (Hicks and Wakefield, 1972; Hicks and Chowaniec, 1978) after a carcinogenic regimen of MNU, and when they occur together with transitional cell tumours, they are regarded as squamous cell carcinoma.

The rough endoplasmic reticulum of the transitional cells of the tumours were fairly well developed and presented a picture suggestive of secretory activity. It may be that, like other malignant or transformed cells, they secrete high levels of enzymes that degrade proteins (Rapin and Burger, 1974; Nicolson, 1976). In addition, these cells would also seem to be engaged in a profuse production of glyco- calyx. The latter is evident as a thick layer on the free surface of the superficially placed cells of the transitional cell tumours. A comparison of figures (116, 120, 130, 133, 139) clearly indicate the vastly increased production of the glycocalyx in the gross tumour compared with the early neoplastic urothelium at 12 weeks post MNU. The microvilli in the tumours are extremely pleomorphic and this feature has often been described by workers in this field, as morphological markers of irreversible hyperplasia committed to neoplastic transformation (Arai et al., 1974; Hicks and Wakefield, 1976; Jacobs et al., 1976; Merk et al., 1977; Newman and Hicks, 1977; Hicks and Chowaniec, 1978; Shirai et al., 1978). In the present investigation where urothelia have been examined at various intervals following a carcinogenic regimen, pleomorphic microvilli appear in abundance late in the series, on obviously transformed cells or transitional cell carcinomas. Pleomorphic microvilli have been observed in carcinomas of very many organs including breast (Spring-Mills and Elias, 1975), cervix (Williams et al., 1973), colon (Kahan et al., 1976) and liver
Ogawa et al., 1979). In view of this, the present author's view is that, instead of being a marker of impending neoplasia, they are characteristic of gross tumours. The more significant finding would seem to be that already mentioned in the urothelium at 12 weeks post-MNU where microvilli first begin to become pleomorphic. This may be the feature to look for, along with others discussed earlier, as an early diagnostic criterion of irreversible hyperplasia and neoplastic transformation. These criteria collectively assume great importance if the results of the rat model are to be applied to human urothelia.

A report apparently contradictory to the above suggestions regarding pleomorphic microvilli and cancerous tissue exists in the literature (Fukushima et al., 1981a, b). By scanning electron microscopy, they claim to have found pleomorphic microvilli on rat bladder urothelial cells of reversible hyperplastic lesions which do not necessarily progress to carcinoma. Several criticisms can be levelled against this claim, the most important being that they have not positively identified or established the nature of the cells with the pleomorphic microvilli by other means such as parallel transmission electron microscopic study. This is crucial because it is known that, after acute damage by toxic substances, the urothelium is invaded by various elements of the connective tissue and reticulo-endothelial system. Focal damage and ulceration adjacent to relatively undamaged areas are frequent in such experiments (Wakefield and Hicks, 1973; Cox and Irving, 1976). Moreover Wakefield and Hicks (1973) and Hicks and Chowaniec (1978) have described phagocytic cells in the urothelium during necrosis after toxic damage. Fukushima et al. (1981a, b) admit the occurrence of ulcers in all their experimental urothelia following injection of
cyclophosphamide, surgical incision, formalin instillation and freeze ulceration. It would, therefore, seem very likely that the microvilli they observed by scanning electron microscopy were those of macrophages or other phagocytic cells.

The significance of the intracytoplasmic and other lumina deep in the urothelium observed at 24 weeks post-MNU would also appear to be limited as a marker of neoplastic transformation. They occur rather late in the present carcinogenesis experiment. Intracytoplasmic lumina displaying pleomorphic microvilli and copious glycocalyx were present not only in the superficially placed layer of cells of the papilloma but also deeper in the cells of the adjacent layer. In addition, supernumerary lumina were also detected in the intercellular spaces within the hyperplastic urothelium as described earlier in this thesis. They appear to be formed independently but it would seem that both intracytoplasmic and intercellular luminae become continuous with the extracellular space. The exact significance of these formations is not clear but probably they are a manifestation of the increased metabolic activity of the tumour cells. Intracytoplasmic lumina have been identified by both light and electron microscopy by previous workers in human and canine "spontaneous" bladder carcinomas and also in experimental bladder carcinomas in rats induced by FANFT (Tannenbaum et al., 1970; Alroy et al., 1979). A survey of the literature shows that intracytoplasmic lumina are frequent in a wide variety of epithelial carcinomas (Wellings and Roberts, 1963; Sagebiel, 1969; Wang, 1973; Dees et al., 1976; Johannessen et al., 1978). In breast neoplasm it was at first thought to be a promising differential diagnostic criterion between benign and malignant neoplasm (Murad and Scarpelli, 1967; Goldenberg et al., 1969; Gould and Snyder, 1974).
This view has to be confirmed because of reports (Archer and Omar, 1969; Tannenbaum, 1969; Gould and Snyder, 1974) of the occasional presence of such lumina in benign and "borderline" breast tumours and rarely in normal breast.

During the course of carcinogenesis, a number of changes were observed at the junction between the urothelium and the connective tissue. The occurrence of multiple lamina and unclarified or apparently damaged basal lamina at 1 week post treatment was discussed earlier in this section. Chronic inflammatory conditions of mucosal tissues are often accompanied by damage to the basal lamina and in part, this would seem to be due to the passage of inflammatory cells through the laminae densa into the urothelium. This could mean loss of contact between some areas of lamina densa and adjacent basal cells which might lead to the formation of new lamina densa by the basal cells. The lamina densa may therefore appear duplicated. During carcinogenesis this has been seen only in early stages (Tarin, 1967). It may also be noted that Pierce and Nakane (1969) have shown that laminated lamina densa material is deposited as a response to the stimulus of epithelial injury induced by bacteria, chemicals or irradiation. The appearance of multiple lamina is not specific to neoplasia. Small breaks in the basal lamina have been noticed after treatment with carcinogens (Smith, 1972). In rat urothelia such breaks occur after various non-carcinogenic treatments and are associated with basal cell pseudopodia (Wakefield and Hicks, 1973) or projections of basal cell cytoplasm into the lamina propria as noticed in the present study in urothelium at 24 weeks post-MNU. Such projections have been called microinvasion by Sugar (1968) and
have been recorded in a wide variety of tissues and in response to many kinds of treatments (reviewed by Wakefield and Hicks, 1973). It can be induced by both carcinogenic and non-carcinogenic chemicals. It occurs in normal untreated tissues during rapid developmental growth or regeneration and has been observed in several human carcinomas. It has, therefore, been concluded (Smith, 1972; Wakefield and Hicks, 1973) that discontinuities in the basal lamina are not specific to neoplasia and are not a criterion for diagnosis of premalignant or malignant conditions.
CHAPTER IV

SPONTANEOUSLY OCCURRING TUMOURS IN HUMANS

General Introduction

The causative factors in cancer of the human bladder are largely unknown. Only a very small proportion of bladder cancer in man is of known etiology; that is in cases where there had been exposure to a few specific chemicals such as β-naphthylamine used in dye industry. Effects are being made in several laboratories to identify bladder carcinogens in the complex environment. Causative factors may exist in the diet, food additives, drugs, tobacco smoke and in the air. One of the main aims of early experimental work in animals was to identify possible bladder carcinogens in man. Rats have been used extensively for this purpose but in some ways, it has not been particularly favourable since it is not susceptible to carcinogenesis by the few chemicals that are known to be carcinogens for human bladder (Hicks et al., 1976). For example, the naphthylamine, which was implicated in human bladder cancer does not produce a typical malignant condition in the rat (Bonser et al., 1952). Genetic differences probably play a role in determining the susceptibility of different species to carcinogens and may involve differences in the activity of drug metabolizing enzymes among different species. The use of complete and direct-acting carcinogens such as MNU have therefore obvious advantages as was pointed out in the previous section.

The other principal reason for the experimental induction of bladder tumours in animals was to study the histological and ultrastructural changes produced, and to use the information for diagnostic
and prognostic purposes in humans and, in particular, to detect markers or early signs of neoplastic transformation. Most histological types of bladder tumours seen in man can be reproduced in experimental rats except "carcinoma in situ". In rat models, the progress of tumour development is too rapid for persistent carcinoma in situ to be established (Hicks et al., 1976). The common papillary tumours of the urinary bladder can be readily reproduced in rats by various carcinogens (Cohen et al., 1976; Hicks and Wakefield, 1972; Koss and Lavin, 1971) but it is uncommon to find invasive carcinomas derived from these papillary tumours. According to Alroy et al., (1981) a problem in drawing analogies between the rat model for chemical carcinogen-induced urinary bladder carcinoma and human bladder carcinoma arises from the fact that, in the rat model, progressive squamous cell differentiation occurs during tumorigenesis, whereas in human transitional cell differentiation often persists throughout the clinical course. Moreover, unlike in man, highly malignant tumours and metastases to distant organs are distinctly rare in rats even with the use of the most potent carcinogens (Koss, 1979; Hicks et al., 1976).

Although there, the rat is not an ideal model for the study of human bladder cancer, the experimental papillary tumours of the rat bladder bear considerable histological similarities to human papillary tumours and this chapter examines some of my findings in this respect. Non-papillary urothelial carcinoma in situ cannot be reproduced except extremely rarely (see also Koss and Lavin, 1971), but I have examined these flat epithelial lesions in man for purposes of comparison with the papillary tumours. The importance of these lesions is that recent multiple sources of evidence indicate that most, if not all, invasive
and metastasizing urothelial cancers of man originate from them (Koss, 1979).

The ultrastructure of low grade, non-invasive papillary transitional cell carcinomas of human bladder has been described before using transmission electron microscopy (Kashiwai et al., 1963; Battifora et al., 1965; Fulker et al., 1971). Of these, the first two are of little significance except in a historical sense. The information provided by Fulker et al., (1971) has been largely confirmed and supplemented by more recent workers (Fuse, 1973; Merk et al., 1977; Koss, 1977; Newman and Hicks, 1977; Tannenbaum et al., 1978), who have paid additional attention to the ultrastructural appearance of the luminal membrane of early papillary tumours. Manzarbeitia et al. (1980) and Alroy and Gould (1980) examined the epithelial-stromal interface in early, papillary transitional cell carcinomas whereas Weinstein et al. (1976) and Alroy et al. (1981) were particularly concerned with cell junctions in various grades of carcinomas.

With the advent of scanning electron microscopy in the late 1960's, there has been a spate of publications on the surface morphology of a wide variety of animal and human tissues and cells. The speed of preparation, the ease of processing and the ability to examine large areas attracted a number of workers to use SEM and to develop it as a diagnostic tool for clinical purposes. In the first reported use of SEM to examine human bladder tumours, Fulker et al. (1971) briefly described varying appearances within a single specimen of well differentiated tumours - a pattern of ridges in some areas and microvilli in others. Hodges (1978) also described overlapping topographic features in transitional cell carcinomas and attempted
to identify changes in relation to tumour grade and stage. In the occurrence of microvilli on human papillary tumours, many workers saw a similarity with experimental animal models of carcinogenesis and this feature was readily assumed to be indicative of early neoplastic change (Koss, 1977; Merk et al., 1977; Newman and Hicks, 1977; Tannenbaum et al., 1978; Gilchrist et al., 1980). To others (Jacob et al., 1978; Davies and Hunt, 1981), this suggestion seemed to be based on inadequate appreciation of the ultrastructural features of normal human urothelium. Kjaergaard et al. (1977) also have drawn attention to the similarities between the surface characteristics of tumour cells and prematurely exposed normal cells of deeper layers of normal urothelium. There were more reports (Nelson et al., 1979b; and Jacobs et al., 1981) on the presence of pleomorphic microvilli on tumour cells regardless of the grade of malignancy. From their SEM study of normal, inflamed and neoplastic human urothelium, Price et al. (1980) concluded that no one cell surface appearance was diagnostic of malignancy and suggested further investigations before SEM can establish definitive diagnostic criteria. In the use of SEM for diagnostic purposes, the need for correlation of factors other than surface topography is becoming increasingly recognised (Carter, 1980). Lack of correlated TEM and SEM studies and imprecise use of morphological terms to describe surface features like microvilli, blebs and ridges have, no doubt, led to some of the confusion prevailing in the literature. In human bladder urothelia, more importantly, the range of normal variations in surface features such as that which may occur with age for instance, were not investigated in advance to serve as a data base against which to assess the significance of surface topography in abnormal and neoplastic tissues.
In this section, I have examined the ultrastructural features of cystoscopically atypical urothelia peripheral to grossly visible tumours. As mentioned earlier, there is no counterpart to these in the rat model of experimental carcinogenesis. Moreover, to the best of my knowledge, only one author (Tannenbaum 1976, 1979) has examined in the electron microscope atypical urothelia referred to as carcinoma \textit{in situ} (CIS). Later in this section, I have also attempted to delineate some of the ultramorphological characteristics of low grade papillary transitional cell carcinomas (Grades I and II) and to compare these features with those observed in the papillary tumours induced in rats.

\textbf{Urothelial Abnormalities Peripheral to Grossly Visible Tumours}

\textbf{Introduction}

Urothelial tumours even when low grade have a 50-80\% recurrence rate. This could be the result of incomplete removal of the primary lesion either adjacent to or at a distance from the tumour (Pyrah et al., 1964). The cytopathological characteristics of normal appearing urothelium peripheral to grossly visible tumours have been known for many years. Following the initial observation of Melicow (1952) and Melicow and Hollowell (1952), a number of investigators have pointed out the existence of cystoscopically occult precancerous urothelial abnormalities such as atypical hyperplasia and/or dysplasia and non-papillary carcinoma \textit{in situ} (CIS) in areas of the bladder not involved in obvious tumour (Schade and Swinney, 1968, 1973; Cooper et al., 1973; Elliott et al., 1973; Ahmed et al., 1976; Farrow et al., 1976; Koss et al., 1977; Soto et al., 1977; Heney et al., 1978, Soloway et al., 1978; Koss, 1979; Murphy et al., 1979; Wallace et al., 1979; Utz et al., 1980). The cystoscopic appearance of CIS has been described
as consisting of a reddening of the mucosa with or without a change in the surface texture to give a granular oedematous, mossy or velvet appearance (Melamed et al., 1964; Yates-Bell, 1971; Riddle et al., 1975; Farrow et al., 1976; Wallace et al., 1979).

The following account relates to biopsies collected from areas peripheral to papillary tumours and which were suspected to be instances of intra-epithelial carcinoma.

**OBSERVATIONS**

Type I: A light micrograph of a biopsy specimen adjacent to a visible tumour is shown in Fig. 145. One of the most striking characteristics of this region of the urothelium is the loose and disorderly arrangement of cells. The number of cell layers may vary from two to six and occasionally some of the superficially placed cells appeared to be large and in the process of desquamation. In LM preparations, it was not easy to determine if urothelial cells extended into the submucosa. The generally large size of the nucleus relative to the size of the cells and the lack of intercellular cohesiveness were also obvious.

In electron microscope preparations, it was generally difficult to find the counterparts of the large cells in the process of exfoliating, which were observable in the LM preparations. Presumably, they separated from the tissue and were usually lost during the preparative procedures for both transmission and scanning electron microscopy. Occasionally, such cells were retained in TEM preparations and they presented a picture suggestive of lysis. The nucleus and cytoplasm were vacuolated and often no recognisable cellular components were visible (Fig. 146). In other instances, abnormal mitochondria
were the only organelles detectable. The cell surface as far as it could be made out was smooth and flexible with no trace of asymmetric unit membrane (AUM) patches. In Scanning microscope preparations, these large cells were never observed. SEM pictures invariably showed the irregular arrangement of the deeper cells (Fig. 148).

Transmission electron micrographs of the cells in deeper layers (Fig. 150) demonstrated wide intercellular spaces and the rarity of intercellular junctions of the adherens type. The few that were seen were very small and seemed to be incompletely formed (Fig. 150 inset). The cell borders were smooth and not thrown into many, long, interdigitating processes. The distribution of chromatin in many cells was also unusual and several nuclei contained inclusion bodies. Within the hyperplastic urothelium there were also present a number of dark cells which, apart from their increased electron density, were not ultrastructurally distinguishable from the other cells (Fig. 150). Free ribosomes were noticed amidst the larger particles of glycogen dispersed throughout the cells. Many mitochondria were found but they almost invariably possessed narrow cristae and dense matrix. Scattered profiles of rough endoplasmic reticulum and independent Golgi complexes were present, but no large vacuoles or obviously secretory droplets were noticed. A class of small vesicles, similar to those in the Golgi complexes were seen scattered in the cytoplasm (Fig. 149). These ultrastructural features were seen in cells throughout the urothelial sample.

The interface between the urothelium and the underlying connective tissue presented certain interesting features. The basal lamina was thick and multiple lamina densa was frequently seen (Figs. 147, 151).
In the limited biopsies that were available, not much of the lamina propria was included and it was therefore not possible to examine the disposition of the capillaries and other elements of the connective tissue. In many instances, the basal lamina was uninterrupted (Fig. 147) but there was a rare instance (Fig. 151) when it seemed as though a urothelial cell had actually entered the submucosa. It would be rather difficult to detect this event in light microscope preparations. Finally, there was a significant absence of hemi-desmosomes of the type that normally anchor the basal layer of cells to the basal lamina (Figs. 147, 151). Conspicuous, however, were invaginations of the plasma membrane of the basal border of the basal cells as seen in Fig. 147.

Type II: In other specimens taken from cystoscopically atypical areas peripheral to a grossly visible tumour, the urothelium was found to be hyperplastic with five to seven layers of cells (Fig. 152). The cells at the luminal surface tended to be cuboidal and larger and their cytoplasm as well as nuclei appeared very pale in the light microscope preparations. Much of the thickness of the urothelium was made up of spindle shaped cells with their deeply stained nuclei occupying disproportionately large areas of the cells. Intracytoplasmic luminae were found in superficially placed cells as well as in deeper cells. The sub-mucosal area underlying the urothelium showed several electron lucent patches.

Transmission electron micrographs confirmed that the large luminal cells were generally in varying stages of lysis, presumably prior to desquamation. Apart from a few collapsed, rounded or ellipsoid vesicles and an occasional polymorphic body, the only organelle present was
mitochondria which looked abnormal with electron dense matrices and attenuated cristae (Fig. 153). The flocculent-looking cytoplasmic matrix contained particles of glycogen scattered either singly or in small clumps. Such cells were joined together by junctional complexes which also appeared to be in the process of dissolution accompanying lytic changes in the rest of the cells. Preparations for the scanning microscope were more successful than the previously described specimens in that some of the large superficial cells were retained. The surface topography of these cells was on the whole smooth with sporadic microvilli-like processes (Fig. 154).

The remaining population of cells of the hyperplastic urothelium were uniformly small cells with large nuclei containing prominent nucleoli and coarsely organised heterochromatin distributed throughout. Overall, the nuclei had a coarsely granular look owing to the presence of many particulate components of various sizes; nuclear inclusions were also frequently met with (Fig. 155). In the narrow cytoplasm of these cells, profiles of rough endoplasmic reticulum and several moderately well developed Golgi complex were prominent organelles apart from unusually dense mitochondria as in the previously described lesion. Desmosomes were more frequently met with than in the other type but they were small. The wide intercellular spaces in the urothelium seemed to contain a fine amorphous substance which resembled the ground substance in the submucosal connective tissue (Figs. 155-159). Pinocytotic activity was evident along the lateral borders of the cells (Fig. 156). In places (Fig. 158), the extracellular space within the urothelium was continuous with the underlying connective tissue in the absence of a well defined basal lamina. This figure also illustrates the disorganised appearance of the epithelial-mesenchymal
The epithelial-mesenchymal junction in this urothelial specimen exhibited several abnormal features (Figs. 157-159). It was quite common to find long cytoplasmic extensions from the basal cells into the connective tissue; these were not evident in the light microscope preparations. At the sites of these intrusions, the basal lamina was disorganised and in the adjoining zone of connective tissue, there were only a few short collagen fibrils or fragments of fibrils and hardly any other connective tissue elements. There were also present several electron-lucent patches within the ground substance of the connective tissue, probably corresponding to the light areas in histological preparations. Some of the electron-lucent areas in the electron micrographs were very close to and/or adjoining the basal cell extensions. The basal lamina was indistinct, and in places, irregular networks of lamina densa were observed. The cytoplasmic processes of basal cells were sometimes seen to be enveloped by a basal lamina, but in other cases, they were not. Hemi-desmosomes were seldom noticed.

The large cytoplasmic extensions of the basal cells into the connective tissue were carefully examined and they were found to contain ultrastructural elements as in the rest of the cell. But what may be of special interest was the presence of small vesicles often close to the plasma membrane (Fig. 159). They ranged in diameter from 30 to 60 nm and resembled vesicles in the Golgi complexes (Fig. 156).

The urothelial biopsies seldom included adequate depths of connective tissue and lamina propria and it was therefore not possible to study the disposition and distribution of capillaries and other connective tissue constituents.
DISCUSSION

It is clear from the figures presented (Figs. 145 and 152) that the histological appearance of the abnormalities in urothelial biopsies suspected by the surgeon and pathologist to be carcinoma in situ is variable. Urothelial abnormalities peripheral to visible tumours are known to range from simple hyperplasia to atypical hyperplasia. In both, the urothelium has been reported to consist of seven or more layers, but in atypical hyperplasia, there are nuclear abnormalities in the form of enlargement, hyperchromasia and abnormalities of shape (Schade and Swinney, 1968; Koss et al., 1974, 1977; Daly, 1976; Koss, 1979). The term "atypical hyperplasia" or "atypia" is probably equivalent to the term "dysplasia" used by others for the same observations (Murphy et al., 1979; Wallace et al., 1979). Again, it would seem that the differentiation between atypical hyperplasia and carcinoma in situ (CIS) has often been difficult and has been a matter for personal preference of the observer. Both lesions are usually present in the same bladder and both are capable of progression to carcinoma (Koss et al., 1977). Different histological types of CIS have been described before (Elliott et al., 1973; Yamada et al., 1975; Ahmed et al., 1976; Farrow et al., 1976; Tannenbaum, 1976, 1979; Koss, 1979; Utz et al., 1980).

In the biopsies that were available for the present study, it was possible to distinguish two types of lesions with different patterns of abnormalities, although both appeared to be invasive. Both differed markedly in morphology from the normal urothelium of elderly subjects. Apart from hyperplasia, the disordered arrangement of cells without definite layers and the absence of any progressive cytodifferentiation from the basal layer to the surface
were the most obvious differences. In both types, however, a few superficially placed cells appeared larger and cuboid but electron microscopic examination established that they were undergoing lysis, presumably prior to being lost in the bladder lumen.

In what I have called type I lesion, the larger size of some cells facing the lumen was probably caused by vacuolization and lytic swelling; there were no ultrastructural features to suggest that these cells were the counterparts of the large differentiated superficial cells of normal urothelium. That these cells were in the process of desquamation was further indicated by the failure to find them in many light and TEM preparations and never in any of the SEM preparations that were made.

At the ultrastructural level, one of the striking features of the cells comprising this lesion was the scarcity of normal desmosomes. Such junctions were not only rare but when present, they were small and incomplete. The lack of desmosomes can account for poor cohesion between cells and the suspected fragility of the urothelium. This finding provides an explanation for the observations of some of the workers in this field (Melamed et al., 1964; Elliott et al., 1973; Ahmed et al., 1976; Farrow et al., 1976; Utz et al., 1980) that the non-papillary in situ carcinoma denudes unusually easily or that the cells from transition cell carcinoma in situ of the urinary bladder exfoliate readily. The ultrastructure of the cells with their moderately well-developed endo-membrane system is interpretable as indicative of the synthesis of some secretory proteins. Even though there were no visible manifestations of secretory activity and no large vacuoles or secretory droplets, it is possible that the cells were in fact involved in the synthesis and discharge
of secretory enzymes. This assumption is based on the presence in cytoplasm, and often close to the plasma membrane, of small vesicles ultrastructurally akin to those in the Golgi complexes.

The coarse nature of the chromatin in most cells of the hyperplastic urothelium may be because they were in prophase stage of mitosis, and this in turn, may be a reflection of a generally decreased generation time of these cells. Mitotic figures were in fact observed in the specimens of CIS examined by Tannenbaum (1979). The significance of the occurrence of dark cells between light cells is not clear. Tannenbaum et al. (1976) have also reported such cells in CIS but, there are reports of dark cells in normal human bladder urothelium (Fulker et al., 1971; Jacob et al., 1978; Tannenbaum, 1979), as well as in well differentiated and poorly differentiated papillary carcinomas (Fulker et al., 1971): the status of the dark cells has been discussed elsewhere in this thesis.

In the type I lesion, the basal lamina seemed to be intact for the most part and in many areas it was much thicker with more than one lamina densa. Hemi-desmosomes, as in normal urothelia, were absent and this feature was in keeping with the scarcity of desmosomes generally which, as pointed out before, can contribute to the lack of cohesion between cells. The occurrence of prominent invaginations of the plasma membrane along the basal border facing the lamina propria is unusual and, in their size and depth, they appear to be different from the pinocytotic activity that is known to occur along the lateral borders of urothelial cells (Jacob et al., 1978). The significance of these large invaginations is unknown but one may speculate that these formations are related to the release of secretory proteins/enzymes synthesised by the cells. This assumption
requires to be confirmed but a factor in favour of such a hypothesis is the rare instance where a urothelial cell was apparently located in the submucosa. The secretory product envisaged (a degradative enzyme) may have enabled the cell to degrade the basal lamina and traverse this barrier. This aspect will be considered again in this chapter. The lack of deeper biopsies precluded a search for further examples of such cell migrations. The invaginations noted along the basal border of basal urothelial cells may be the initial manifestation of a mechanism for exteriorisation of hydrolytic enzymes leading to the formation of micro-destruction zones around the neoplastic cell. The existence of such zones around various cancer cells has been established by light and EM studies (Birbeck and Wheatley, 1965; Tarin, 1967, Woods and Smith, 1969; Chowaniec and Hicks, 1977).

In the type II lesion also, the superficially placed cells seemed to be undergoing cytolysis and about to be desquamated from the urothelium. The presence of collapsed apical cytoplasmic vesicles, polymorphic bodies and junctional complexes as in normal superficial cells suggest that, unlike in the type I lesion, at least some of the large cells in the process of lysis in the type II lesion are comparable to the superficial cells of the normal urothelium of elderly subjects. By this criterion, it would seem that this lesion is a more recently formed one than the other type already discussed.

The more frequent occurrence of desmosomes, even though they were small, indicates that the intercellular cohesiveness in this atypical hyperplastic urothelium is not so low as in the type I lesion, at least at this stage in the development of the abnormality. This lesion also differed from the other in the presence of intracytoplasmic
lumina in cells at different depths. Moreover, the cells were spindle shaped and possessed a few interdigitating cell processes along their borders.

The nuclei with their inclusions and coarse chromatin appeared very much similar, indicating prophasic organisation and shorter generation time. At first glance, the untrastructure of the cytoplasm also seemed similar to that in the cells of the other lesion, with mainly rough endoplasmic reticulum and several independent Golgi complexes. Although no quantitative analysis was made, it seemed as though both these organelles were more extensive and better developed in the present instance. Also noticeable in this tissue was an amorphous substance which occupied much of the intraurothelial but extracellular (intercellular) spaces and which also seemed to be continuous with a similar ground substance in the adjoining connective tissue.

In the type II lesion under discussion, there were considerable alterations in the epithelial-stromal interface. These disturbances and alterations were more marked than in the type I lesion. The alterations in the basal lamina consisted of ramification in places of two, three, or more lamina densa and in other locations of disruption of its continuity. These alterations were, in the main, associated with large cytoplasmic extensions of the basal cells. The lack of a distinct "basement membrane" and the presence of "pseudopods" in basal cells of CIS were also observed by Tannenbaum (1979). As stated above, some of the cytoplasmic extensions of the basal cells were not enveloped by a basal lamina and therefore the discontinuities in the basal lamina must be regarded as genuine gaps in the interface. As far as could be judged by ultrastructural appearance, it seemed as though there was destruction of the basal
lamina in these areas, although an intact basal lamina was present in other areas. It is noteworthy that it was often adjacent to the areas of apparent destruction of the basal lamina, that the electron-lucent patches occurred in the submucosa.

The occurrence of gaps in the basal lamina and associated electron-lucent areas in the adjacent submucosa together with the concomitant presence of cytoplasmic extensions of basal urothelial cells prompt speculation that some hydrolytic enzymes may be released from the cytoplasmic processes for the destruction of the basal lamina and the adjoining connective tissue elements. In this context, the presence of small vesicular components in the cytoplasmic extensions may be significant. As described earlier, these vesicles are of the same size and ultramorphology as those present at or in the vicinity of Golgi complexes and the proposition that they are the carriers of hydrolytic enzymes synthesized by the rough endoplasmic reticulum to the cell exterior, is an attractive one. The presence of small empty-looking vesicles (primary lysosomes) containing acid phosphatase has been demonstrated in normal bladder urothelial cells of the elderly (Jacob et al., 1978), but the enzyme most likely to break down basal lamina and the adjoining connective tissue is collagenase.

The idea that cells undergoing neoplastic change may synthesize and discharge enzymes has been proposed by a number of workers (Holmberg, 1961; Sylven, 1962; Taylor et al., 1969; Keiditsch and Strauch, 1970; Dresden et al., 1972; Unkeless et al., 1973). More relevant to the present discussion is the suggestion by David and Mangakis (1963), Woods and Smith (1970), and Smith (1972) that destruction of the "basement membrane" results from enzymes produced
by the neoplastic basal cells. The proposition advanced, therefore, is that cells of the lesions examined, type II more than type I, produce and discharge collagenases to bring about the dissolution of basal lamina and adjoining connective tissue and thereby enable their entry into the submucosa. The synthetic machinery for the production and transport of hydrolases (i.e. RER and Golgi apparatus) is well manifested in the epithelial cells under discussion. However, this aspect requires further study and clarification in further samples of tissue including cytochemical investigations to establish the lysosomal nature of the vesicles. Tissue collagenases have been found in amphibian and mammalian tissues undergoing growth or remodelling. High enzyme activity has been found in the tail fin of tadpoles during metamorphosis and in mammalian uterus undergoing rapid reorganisation following pregnancy. Current speculation (cf. Croft and Tarin, 1970; Constantinides, 1974, Ghlsson et al, 1977) is that cell lysosomes may contain collagenase. There are recent reports of increased collagenase activities in human and animal neoplasms (Dresden et al., 1972; McCorskerry et al., 1975; Kuettner et al., 1977) and recently Wirl and Frick (1979) have demonstrated collagenase in human bladder cancer by biochemical methods. Their assays revealed high collagenase activity only in the case of deeply infiltrating tumours but not in superficially infiltrating tumours and they suspected the enzyme to be a product of stromal elements rather than the tumour epithelial cells. More work is needed in this area and it is the present author's view that with more sensitive assay methods using a larger number of specimens, it might be possible to detect collagenase in superficially invasive tumours as well. In the present study, the cyto-differentiation observed in such cells show that they are endowed
with the machinery for the synthesis of exportable proteins.

As pointed out earlier, there are no descriptions at the EM level of superficially invasive urothelial abnormalities peripheral to grossly visible tumour apart from that by one author (Tannenbaum, 1976; 1979). The present ultrastructural study of limited samples of suspected CIS show that although histological variations may exist between different specimens, there are several common features that should enable them to be distinguished as potentially invasive lesions. These include lack or scarcity of adherence junctions, moderately well developed endo-membrane systems in the small cells with high nucleo-cytoplasmic ratio, which form the bulk of the hyperplastic urothelium. Also noticeable are the striking alterations in the epithelial-mesenchymal junction involving basal cells, basal lamina and the immediately adjoining connective tissue. These multiple ultrastructural criteria in the specimens analysed suggest that the presence of these lesions is probably ominous, especially since the biopsies came from bladders with superficial papillary tumours. In the clinical setting, two types of CIS have been described (UICC Technical Report, 1981), but there is no knowing how far they correlate with the histopathological descriptions available in the literature and the present ultrastructural account. More closely correlated clinical, histological and ultrastructural studies in the future can clarify the problem of the relationship of benign hyperplasia and atypia of the urothelium, to CIS, and help to distinguish the natural history of different types of CIS.

The identification of preneoplastic lesions is a matter of great importance but the problem is that this distinction cannot easily be made with histological criteria, since many of the lesions may look alike at this level of study. Finer discrimination that is possible
with the transmission EM studies is likely to provide a better clue to
the fate of atypical urothelia and indicate which of them are probably
reversible and benign and which are destined to proceed to invasive
carcinoma. It is suggested that the various manifestations such as
those revealed in the present study are collectively indicative of
preneoplastic lesions. Further collaborative studies of carefully
chosen biopsies may be expected to define the criteria more precisely,
since the EM can reveal abnormalities that are beyond the resolution
of the light microscope.

**Papillary Transitional Cell Carcinomas**

**Introduction**

Until 1973, there was no general agreement on classification and
nomenclature of bladder tumours, when WHO published new rules which
became widely adopted. Many bladder tumours are papillary and in the
generally accepted grading systems (Mostofi et al., 1973; Koss, 1975,
UICC, 1978), the degree of cellular anaplasia forms the basis of
grading. Even so, grading may depend on subjective criteria (cf.
Ooms, 1981) and difficulties can arise in practice to decide whether
a given tumour is grade I or grade II (UICC Technical Report, 1981).

For the present study, the histopathological evaluation of
biopsies was performed by the Department of Pathology, Western General
Hospital, Edinburgh, and only those identified as grade I and grade
II were accepted. The main objectives of this investigation were
(a) to compare the ultrastructural features of these carefully
selected low grade papillary transitional tumours with those of
normal urothelia in elderly subjects, (b) to examine criteria of
early neoplasia in the light of studies in experimental animals, and
(c) to make an attempt to establish ultrastructural features that might serve to distinguish between grade I and grade II carcinomas. Histological findings such as those relating to size and shape of tumour cells and nuclei and their dispositions have been largely confirmed by the electron microscope studies of Battifora et al., (1965) and Fulker et al., (1971), and will not be considered in this account.

**OBSERVATIONS**

**Grade I**

In light microscopic preparations and low magnification scanning electron micrographs, grade I carcinoma appeared in the form of single fronds or stalks. The hyperplastic urothelium was about eight layers thick. The luminal layer of cells were of variable size, some were large and some were small, but those in the deeper layers were small and more or less uniform in size.

The larger cells at the luminal surface resembled the large superficial cells of normal urothelium in the elderly. There were a few round vesicles in the apical cytoplasm limited by symmetrical membrane (Fig. 160). In addition, these cells contained polymorphic bodies, Golgi apparatus and other organelles as in the normal urothelium in the elderly. Likewise, the lateral borders of the large superficially placed cells were joined together at the luminal surface by junctional complexes, although at times, the tight junctions seemed to be rather attenuated. The luminal surface of these cells was variable as in normal urothelia in the elderly with simple, wavy outline and sparsely distributed microvilli or a more scalloped appearance with microvilli at the crests or a mixture of the two (Figs. 160 and 161) as described elsewhere in
in the urothelium of the elderly. On the other hand, the smaller cells at the luminal surface generally showed more microvilli which on the whole appeared uniformly distributed (Figs. 162, 165). Careful examination of the luminal surface in transmission electron micrographs which enabled two features of interest to be discerned were not found in the normal human urothelia of any age group examined in this study. One of these was that the microvilli of the luminal layer of cells - both large and small - were covered with a thin layer of cell surface coat or glycocalyx which however delicate was noticeable in preparations made by routine procedures. The other feature was the occasional appearance of branched microvilli and/or microvilli with variable dimensions such as height and diameter (Fig. 162). Several of the cells exhibiting these unusual features at the luminal surface possessed other ultrastructural features such as an odd polymorphic body, or sporadic rounded vesicles in the apical cytoplasm. The junctional complexes between the large superficially placed cells were similar to that of the normal aged urothelium as already stated. In many instances, the apparently tight junctions seemed short and attenuated. On the other hand, the luminal junction between the small cells seemed more like macula adherens.

The cells in the deeper layers of the urothelium were more or less uniform in size and their state of differentiation was variable between that of the intermediate and basal cells of the normal urothelium in elderly subjects. The nuclei of the cells were also highly lobate, but they often contained inclusion bodies, seldom noticed in cells of normal urothelia. The layering of cells in the hyperplastic urothelium was still more or less regular but some disturbances were noticeable in places. Desmosomes were present between the cells as in the normal urothelium and there were also
variable amounts of glycogen.

In some tumours of this grade, cells with more spherical nuclei and uncharacteristic cytodifferentiation were encountered (Figs. 116, 117) in focal areas. They occurred in isolated sections that were examined during the course of this investigation. The nuclei had a coarsely granular appearance with clusters of granules of different sizes. In one type (Fig. 166) of these unusual cells, the rough endoplasmic reticulum and Golgi apparatus were extensive and well developed, while in the other (Fig. 167) the cytoplasm, especially the perinuclear area, was filled with microfilaments and bundles of microfilaments not unlike those described in the so-called squamous metaplasia in urothelial cells of experimental rats. In both cell types, the mitochondria were mostly spherical with sparse cristae and electron-lucent matrices: some of the mitochondria were obviously undergoing degeneration.

The basal lamina in most places was thin-and continuous and hemidesmosomes were also noticed as in normal urothelium. The basal lamina was for the most part straight and ran parallel to the stroma of the tumour stalk. On occasions, much thicker stretches of basal lamina were observed in which the lamina densa appeared reduplicated several times (Fig. 163). In other instances, the basal lamina appeared thick but rarefied with electron-lucent patches and it closely followed tiny projections from the basal cells (Fig. 164). Also apparent in such micrographs were electron-lucent areas in the immediately adjoining connective tissue and indications of what seemed to be dissolution of collagen fibrils.
Grade II

The grade II carcinoma consisted of broad and multiple stalks of papillae made up of ten or more layers of cells. The cells of the hyperplastic urothelium were pleomorphic and tended to be irregularly arranged. At the luminal surface large cells were scarce; in those that were present (Fig. 169), the apical cytoplasm resembled that of the normal superficial cells in the presence of many rounded vesicles. It was surprising to find occasionally in some cells, elongated or fusiform vesicles, reminiscent of normal urothelial cells. Most of the luminal cells were small and variable in shape. The characteristic appearance of the luminal surface is shown in Figs. 168-170. On the majority of cells, the microvilli were not only prolific but also clearly pleomorphic. In addition, transmission electron micrographs strikingly demonstrated the presence of a thick, electron-dense glycocalyx (Figs. 168, 169). These preparations, it may be re-emphasized, were made by usual routine procedures of fixing and staining. The pleomorphic nature of microvilli could be made out in scanning electron micrographs (Fig. 170) but it was difficult to visualize the rich glycocalyx in such preparations. The apical junctions at the luminal surface appeared mostly abnormal in that there were no indications of zonulae occludentes. The cells, however, were held together by desmosome-like junctions (Fig. 168) as in grade 1 papillary carcinoma.

The ultrastructural organisation of the cells comprising the hyperplastic urothelium was simple. There were a few vesicular components in the cytoplasm of the cells situated in the more peripheral layers. The cells generally contained smaller Golgi apparatuses, sparse profiles of RER, and widely distributed mitochondria. Polymorphic bodies were seen occasionally but there was
variation in this and the other features stated above from cell to cell. The nuclei of the cells also varied in size and shape, and showed fewer indentations of their envelope: inclusion bodies were often noticed in them. Heavy deposits of glycogen were present in some of the cells and others contained smaller amounts. Both alpha and beta particles of glycogen were noticed.

Close to the luminal surface, junctional complexes were visible, but as already mentioned, they were small and made up of adherens type junctions only. The desmosomes observed were also small. The basal lamina of the tissue was frequently thickened with multiple lamina densa as in the grade 1 tumours.

Another feature of the grade II carcinoma was the presence of intracytoplasmic lumina (Fig. 168) not only in cells of the superficial layer but also in those of lower layers. The lumina also showed variable numbers of pleomorphic microvilli. Intracytoplasmic lumina were not detected with certainty in scanning micrographs owing to the presence of the long and pleomorphic microvilli (Fig. 170).

**DISCUSSION**

There have been very few attempts to study the ultrastructure of human bladder carcinomas (Battifora et al., 1965; Fulker et al., 1971). The situation in this respect is much the same as with normal human bladder urothelium. More recent work with the TEM on human bladder tumours have been concerned with certain specialised aspects such as permeability barrier and intercellular junctions (Weinstein et al., 1974); Merk et al., 1977; Alroy et al., 1981), structure of plasma membrane (Weinstein, 1976) and the interference between epithelium and stroma (Alroy and Gould, 1980). In other TEM studies dealing
primarily with experimental animal models, there are brief references
to human tumours for purposes of comparison (Koss, 1977; Newman and
Hicks, 1977). As stated elsewhere in this thesis, there are also
several mainly SEM studies in which human bladder tumours have been
examined for evaluation of surface features in terms of information
available from animal models and/or for diagnostic purposes (Kjaergaard
et al., 1977; Hodges, 1978; Tannenbaum et al., 1978; Nelson et al.,
1979b; Price et al., 1980; Gilchrist et al., 1980; Jacobs et al., 1981).

The histological criteria, such as that the low grade papillary
carcinomas resemble the normal bladder urothelium with transitional
cells still capable of normal differentiation and orderly layering
of cells, but are thicker with many more cell layers, have been validated
by the present transmission electron microscope study as well as those
of previous workers like Fulker et al. (1971). There were present
occasionally in these tumours, especially in grade I, large cells at
the luminal surface with a few round vesicles in the apical cytoplasm,
polymorphic bodies, and other features as in normal urothelium.

In early scanning electron microscope studies Fulker et al.
(1971) noticed irregularities in the surface ultrastructure of low
grade papillary carcinomas and described them as being caused by the
presence of small and large cells. What was, however, not known at
that time is the fact that irregularities in the surface occur in the
normal urothelium of elderly subjects as well. The occurrence of
thick and thin regions with large differentiated cells and small
undifferentiated cells at the luminal surface was first reported by
Jacob et al. (1978) and has now been confirmed by the present in-
vestigation. Bladder cancer is infrequent in children and adults
but increases with age in an exponential fashion after the fifth
decade (Clayson, 1975; Williams, 1975). The appropriate normal
urothelium for comparison with the tumours therefore would be the aged urothelium. As described elsewhere in this thesis, the luminal surface of the urothelium of the elderly may be described as microvillous both in the thick and thin regions - in the former region owing to a lower level of differentiation in the large superficial cells, and in the latter due to the undifferentiated nature of the small cells lining the lumen. The different nature of the two types of cells - both of which display microvilli on their surfaces - will not be evident in a study of the surface of normal aged urothelia by scanning electron microscopy, although the variable sizes and shapes of the cells may be noticeable. The flexible and microvillous surface feature of the aged human urothelium contrasts sharply with the well known scalloped pattern of microridges on the flat polygonal cells of rat urothelium. The presence of microvilli and the virtual absence of specialised asymmetric membrane plaques at the luminal surface appear to be consequences of ageing, as pointed out in an earlier chapter. If an aged urothelium with these features were examined by an inexperienced worker in an exclusive scanning electron microscope study, chances are that the urothelium may have been erroneously interpreted as neoplastically transformed. This possibility arises from the fact that in the animal model of carcinogenesis, it was once suggested that the appearance of microvilli on non-polygonal cells at the luminal surface may be an early change in neoplastic transformation. A straightforward analogy with the rat model would hence have been misleading.

During the last decade, several SEM investigators have described the presence of microvilli on the surface of human papillary tumours (Fulker et al., 1971; Newman and Hicks, 1977; Hodges, 1978; Tannenbaum
1979; Jacobs et al., (1981). In analogy with observations in experimental animal carcinogenesis, many of the above workers have suggested that the mere presence of microvilli at the luminal surface is an early change in neoplastic transformation. One factor which appears to have contributed to this suggestion was the implicit assumption hitherto of many workers that the ultrastructure and especially the surface features of human urothelium were identical to those of the rat and that the ultramorphology remained unchanged throughout the life span. The present study as well as that of Jacob et al. (1978) make it clear that interpretations based exclusively on SEM observations of microvilli at the luminal surface may be misleading.

Low grade papillary carcinomas, undoubtedly possess microvilli as shown in the present study and in previous studies, but since no instance of hyperplasia has been found in normal aged urothelia, a distinction between normal aged urothelium and abnormal urothelium can be easily made by TEM as well as histological analyses. At the ultrastructural level, a further distinction between the two seems to lie in a quantitative difference in the microvilli rather than the mere presence or absence of microvilli per se. In low power transmission electron micrographs, the microvilli at the luminal surface of aged urothelium can be seen to be generally sparse and unevenly distributed, as described in an earlier chapter. On the other hand, microvilli on the luminal surface of grade 1 papillary carcinoma are generally more plentiful, more regular and more uniformly distributed. The distinction between these surfaces is not difficult especially in low power TEM preparations.

A loss of differentiation from the base to the surface and a lack of a specialised luminal plasma membrane as shown in the aged human urothelium may conceivably occur in human urothelium accompanied
by hyperplasia. Such situations have been found in urothelia of experimental rats subjected to toxic treatment (Hicks, 1976, and others) and if this condition were to occur in humans, as for example following a long fractionated heat treatment (Jacob et al., 1982), it should be possible to discriminate between what is possibly a benign, reversible state and an irreversible neoplastic state.

Careful examination of a large number of transmission micrographs of grade I tumour cells - both large and small cells - have in variably shown a delicate but definite glycocalyx. This material could be made out in preparations processed by routine procedures (that is, without special staining) and was never noted in similarly prepared normal human urothelia or in the regenerating hyperplastic urothelium described by Jacob et al. (1982). The presence or absence of glycocalyx has not been critically examined in cases of benign hyperplasia in experimental animals, and it should be of interest to clarify this in TEM rather than SEM studies. But, as pointed out elsewhere in this thesis, there have been suggestions from experimental studies in animals using transmission electron microscopy that an ultramorphological change - probably reflecting qualitative change - occurs in the glycocalyx during neoplastic transformation.

An additional feature relating to microvilli that was manifested but only sporadically, in the grade I tumours was pleomorphism. The luminal surface of any normal bladder urothelium does not have pleomorphic microvilli. In many SEM investigations in the past, microvilli, in low grade papillary tumours have been described as not only numerous but also pleomorphic. A careful analysis of this feature in the two carefully selected grades of tumours in the present study has shown that large-scale pleomorphism of microvilli is predominant in the grade II carcinoma, although again, as with many other features,
there is some variability in different biopsies of this grade, and also in different areas of a given sample. Attention has been drawn in the past (Hodges, 1978; Jacobs et al., 1981) to the continuum of changes both within and between tumour grades and continuum of changes related to various features in a given tumour. The existence of a sampling problem is well known in transmission electron microscopy but examination of a reasonable number of mm² tissue blocks at random from a given biopsy should reveal the presence of the pleomorphic microvilli if they actually occur in the biopsy. A significant outcome of this investigation is the demonstration that it may be possible to distinguish between grades I and II papillary carcinomas by transmission electron microscopy. There is a second feature that may be looked for in parallel, and that is the prominent glycocalyx in routine TEM preparations, which is so much more electron-dense and conspicuous in comparison with that observed in grade I. This additional feature, unfortunately, is not discernible in the SEM: SEM preparations of these tumours would seem inadequate for the detection and evaluation of glycocalyx. Fragmentary reports of a conspicuous glycocalyx in papillary carcinomas of human bladder are present in the accounts of some workers (Koss, 1977; Merk et al., 1977; Newman and Hicks, 1977; Alroy and Gould, 1980) but they did not correlate it with the grade of the carcinoma.

From the above discussion, it would seem that the usefulness of scanning electron microscopy is somewhat limited in discriminating crucial features of human bladder carcinomas, and this fact together with the continuum of changes within a given sample probably accounts for some of the confusion in the literature and frustration of some workers (for example: Kjaergaard et al., 1977; Price et al., 1980),
who concluded on the basis of SEM studies that there is no tumour-specific surface morphology or that there is no one cell surface appearance that indicates malignancy.

In both grade I and II tumours, nuclear inclusions of very low electron density were common. These entities were also noticed in normal urothelial cells. Some workers who have obviously not made a detailed study of normal urothelium have implied (Manzarbeitia et al., 1980) that intranuclear bodies are characteristic of the neoplastic cells of early urothelial tumours.

The changes in the basal lamina such as thickening and reduplication or the occurrence of rarefied basal lamina penetrated by minute cytoplasmic processes have been considered elsewhere in this thesis and their significance was discussed at length in the context of experimental carcinogenesis in rats. Opinion seems to be divided on this question and on their relevance to the progression of carcinoma, as many of these changes have been met with in healing or regenerative processes following various kinds of damage unrelated to neoplasia or malignancy. However, although the various changes mentioned above at the urothelial-mesenchymal junction resemble those observed in other situations, more careful analysis of these in future may reveal subtle differences that may enable one to distinguish those that are related to developing neoplasia and/or impending invasion from others related to benign changes.

Intracytoplasmic lumina have not generally been detected in transitional cell carcinomas of human bladder; the only reports so far are that of Tannenbaum et al., (1970) and Alroy et al. (1979). As pointed out elsewhere in this thesis, they have been reported in several other tumours as well as benign and normal tissues. Their true significance, therefore, remains unclear although if human bladder
is considered, they must be regarded as pointing to neoplasia in as much as that they have been reported only once in the normal bladder urothelium (Monis and Dorfman, 1967).

Examination of the connective tissue and deeper regions of the tumour urothelium has been inadequate because of the lack of suitable, i.e. deep, biopsies. It is therefore not possible to state whether there are any changes in the subepithelial blood capillaries of the low grade tumours, comparable to that noted in the experimental animal (discussed elsewhere in this thesis). In the light of the evidence from animal models, it would seem important to examine the submucosa more carefully and to evaluate any disturbances that might be present.

Another noteworthy feature that has emerged from this study is the presence in a grade I carcinoma of two cell types with unusual cyto-differentiation. In one type, large quantities of microfilaments and abundant ribosomes were observed; in some instances large areas of cytoplasm was occupied by the filaments. This condition recalls to mind the condition of urothelium described as foci of squamous metaplasia in experimental rat bladder tumours where they are thought to play an important role in the development of invasive carcinomas. The occurrence of cells containing bundles of tonofilaments was reported once before in the TEM studies of low grade transitional cell carcinomas of human bladder (Battifora et al., 1965). Cells rich in microfilaments were also found in a grade II carcinoma. In the other type, the altered differentiation of the cell with extensive membranous organelles like the rough endoplasmic reticulum, seemed to be geared for large scale production of secretory proteins - perhaps hydrolytic enzymes. Many cells secrete proteases and there is some evidence that some neoplastic cells secrete more than their non-neoplastic counterparts. Among these enzymes are collagenases
(Gould and Battifora, 1976). It is doubtful whether such foci of altered cells would be easily detected in conventional histological preparations. Foci of altered cells in an otherwise uniform population of cells may mark the emergence of a subclass of cells with more aggressive properties and hence to a progression of the carcinoma.
CHAPTER V

INTERACTIONS OF LECTINS WITH NORMAL HUMAN UROTHELIA AND PAPILLARY CARCINOMAS

Introduction

At the present time, there is considerable evidence which indicates that fundamental changes occur at the cell surface during neoplastic transformation and that these changes determine most of the properties of tumour cells. These properties include loss of growth control, expression of new antigens, invasiveness, decreased cellular adhesion and metastasis. The study of the properties of transformed cells has been greatly facilitated by the use of cell culture systems, and the increasing understanding of the architecture and function of the cell surface is due in large part to a class of proteins called lectins which bind to sugar molecules much as enzymes combine with their substrates and antibodies combine with their antigens.

Some of the lipid molecules and proteins that comprise the plasma membrane carry branching chains of sugar molecules (oligosaccharides) that extend from the cell surface: they are glycolipids and glycoproteins. More than 100 monosaccharides are known in nature, and they can combine with one another in many ways. Only about nine of these monosaccharides have been detected in the glycoprotein and glycolipid of the cell surface, and they are usually combined in oligosaccharides of no more than 15 sugar units; the number of possible combinations is nevertheless still very large.

Lectins bind non-covalently to specific carbohydrate groups without modifying them chemically. Binding is reversible and all lectins have more than one specific carbohydrate-combining site.
When lectins which have multiple combing sites bind to saccharides, they serve as cross-linking agents to interconnect many cells causing them to agglutinate. Biologists have made the most conspicuous use of lectins as probes in studies of cell surface structure and function.

Lectins: Lectins are found not only in plants but also in invertebrates and even in mammalian tissues. Liener (1976), who has reviewed the sources of lectins, has suggested that the term lectin be used as a generic term to denote all sugar-specific proteins with designations such as phytolectins, zoolectins, and mycolectins to denote their plant, animal and fungal origin respectively. Lengthy reviews on the use of lectins and the results obtained in numerous systems are available (Nicolson, 1974, 1976, 1980; Rapin and Burger, 1974; Brown and Hunt, 1978).

Liener (1976) has considered at length the properties of phytolectins and what follows is a brief account of the lectins used in the present work.

1. **Concanavalin A (Con A):** The lectin from *Canavalia ensiformis* (Jack bean) is the most extensively studied plant lectin. It was first isolated in quantity by Sumner and Howell (1936). The primary, secondary and tertiary structure of Con A has been the focus of research in several laboratories. Con A is composed of identical 25,500-molecular-weight, asymmetric subunits (protomers) arranged in dimers, tetramers, and higher-molecular-weight forms depending on the pH; below pH 5.6 Con A exists in solution as a dimer molecule containing two subunits (Becker et al., 1971); above pH 5.6 Con A forms a tetramer, and at pH values above 7 the tetrameric form further associates, forming higher-molecular-weight aggregates (McKenzie et al., 1972). Beside the pH, the activity of Con A is markedly influenced by temperature.
(Hunt et al., 1975), and chemical modifications (Gunther et al., 1973). A tentative primary sequence for Con A was determined by Edelman et al. (1972); Con A is a single polypeptide chain containing 238 amino acid residues.

Although Con A interacts specifically with α-D- mannose, α-D-glucose, and α-N-acetyl-D-glucosamine residues located at the terminal, nonreducing ends of polysaccharide chains, it is now evident that Con A is also capable of binding mannose residues located in the interior of the molecule (Chase and Miller, 1973; Goldstein et al., 1973; Kaifu et al., 1975). This probably explains why a number of glycoprotein and glycopeptides are strong inhibitors of Con A despite the fact that their terminal position is blocked with sialic acid (Chase and Miller, 1973; Edelman et al., 1972).

2. Wheat Germ Agglutinin (WGA): WGA was first isolated and purified from wheat germ (Triticum vulgare) by Burger and Goldberg (1967). WGA exists as a 35,000-molecular-weight dimer in neutral pH buffers with two binding sites for inhibitory saccharides; at lower pH, the molecule exists as a 17,000-molecular-weight monomer (Nagata and Burger, 1974). The X-ray crystallographic structure for WGA has confirmed the previous studies, which showed that the overall WGA molecule is a dimer of two identical subunits in close association with each other across an exact two fold axis. Each monomer contains four structurally similar domains of approximately 41 amino acids each (Wright, 1974; Wright et al., 1974).

WGA binds saccharides and oligosaccharides containing D-GlcNac residues such as (D-GlcNac)$_2$ and (D-GlcNac)$_3$ (Allen et al., 1973; Nagata and Burger, 1974). (D-GlcNac)$_3$ is approximately 300 times as potent a WGA inhibitor as D-GlcNac (Allen et al., 1973). Le Vine et al. (1972) proposed that WGA binds only one molecule of D-GlcNac
per molecule of WGA, but WGA has another saccharide-binding site for N-acetylneuraminic acid which may explain the decrease in WGA agglutinability of cells after neuraminase treatment (Burger, 1969; Nicolson, 1973).

Oikawa et al. (1973) have made the interesting observation that WGA can block the fertilization of hamster eggs by combining with sites in the outer region of the zona pellucida. This effect was believed to be due to the formation of cross-linkage of these sites so as to prevent the dissolution of the zona pellucida by capacitated spermatozoa.

3. **Phytohaemagglutinin (PHA):** PHA was isolated by Rigas and Johnson (1964) from red kidney beans (*Phaseolus vulgaris*). The attempts by Lis and Sharon (1973) to separate the various components responsible for the erythroagglutinating, leucoagglutinating and mitogenic activities of PHA have been often contradictory and to some extent confusing (Liener, 1976). The air appears to have been cleared somewhat by the recent work of Yachnin and Svenson (1972) and Miller et al. (1975), who found that all of the diverse biological activities of the kidney bean could be accounted for by a family of five heterogeneous proteins. Each of these consists of isomeric, non-covalently bound tetramers which are made up of two different subunits, designated as L and R. Each of these subunits has approximately the same molecular weight, 34,000, but differs to some extent in their amino acid sequence. More important, however, is the fact that the L subunit has strong mitogenic activity and a high affinity for receptors of lymphocyte membranes, but little or no affinity for those of erythrocytes. Conversely, the R subunit has a high affinity for erythrocyte membrane receptors, but little for those of lymphocytes. As a
consequence of this difference in specificity, the tetramer with 4L subunits (L - PHAP) is a potent leucoagglutinin with low haemagglutinating activity. These hybrid tetramers with two or more R subunits (2R - 2L, 3R - 1L, and 4R) exhibit potent haemagglutinating activity but modest leucoagglutinating activity and are referred to collectively as H-PHAP. The hybrid molecules (3R - 1L, 2R - 2L, and 1R - 3L) have been found to be mitogenic, indicating that both L and R subunits are required for this kind of activity.

Both L-PHAP and H-PHAP were found to bind saccharides and oligosaccharides containing D-GalNAc residues (Rigas and Head, 1969, Miller et al., 1973).

4. Pokeweed mitogen (PWM): One of the most potent plant mitogens is found in the pokeweed (Phytolacca americana). Waxdal (1974) isolated from crude extracts of this plant five different mitogenic proteins, each of which had distinct physico-chemical properties and biological activities. One of these, designated as Pa-1, was the most potent haemagglutinin and mitogen and appeared to be a polymer of 22,000-molecular-weight subunits. The other four mitogens were monomers with molecular weights ranging from 19,000 to 31,000. One of these, Pa-2 was active only on the T (thymus dependent) class of lymphocytes, whereas Pa-1 mitogen was capable of stimulating B (bone marrow dependent) lymphocytes as well as T-cells (Waxdal and Basham, 1974).

PWM was found to specifically bind saccharides and oligosaccharides containing (D-GlcNAc)₂ (Aizawa and Kurimoto, 1979b).
Interaction of Lectins in Different Cell Populations

(1) Lectins in embryological development and ageing: Lectins have provided evidence that the cell surface is altered in the course of embryonic cell differentiation. Moscona (1971) and Kleinschuster and Moscona (1972) first demonstrated that the effect of Con A on the nerve cells in the retina and hepatic cells of the chick embryo depended very much on their embryonic age. Dispersed cells from young (8-9 days) embryo were agglutinated rapidly and massively by Con A. The effect changed as the cell differentiated and matured, so that 20-day cell agglutinated only very poorly. Also Weiser (1972) has found that Con A markedly agglutinated isolated epithelial cells from the intestine of human foetus, but not from the intestine of the adult. With chick embryonic cells, receptors for different lectins react quite differently during development. Comprehensive reviews are available on interactions of lectins with eggs, spermatozoa and embryonic cells of a variety of organisms (Nisolson, 1974; Brown and Hunt, 1978).

(2) Lectins in in vitro ageing at the cellular level: Human diploid fibroblasts have been extensively studied as an in vitro model of ageing at the cellular level and much work has been reported on DNA, chromatin and enzyme changes which accompany proliferative decline on the cells. But relatively little is known about the changes on the cell surfaces. SEM observations by Bowman and Daniel (1975) and freeze-fracture analyses by Kelley and Skipper (1977) have indicated changes in cell surface topography and membrane organisation. There are also some reports on antigenic changes in cell surfaces associated with cell senescence but some of the results are contradictory (Sasportes et al., 1971; Brautbar et al., 1972; Goldstein and Singal
Selective agglutination of suspended cells by lectins has been used by some workers to detect cell surface alterations. For instance, Yamamoto et al. (1977) noted a decrease in Con A mediated agglutination in aged chick fibroblasts. With the advent of the lectin mediated, RBC adsorption assay of Furmanski et al. (1972) it became possible to examine cells in monolayers, rather than in suspensions. Using this method Aizawa and Kurimoto (1979a,b) and Aizawa et al. (1980a,b) have been able to demonstrate cell surface changes accompanying ageing in human diploid fibroblasts.

Kelley et al. (1978) have found no major differences in the total number of binding sites per cell of either the early or late passage human embryo fibroblasts when reacted with Con A. However, they found that haemocyanin-labelled binding sites tended to be more clustered on membrane of late passage cells in contrast to more homogeneous patterns of distribution in early passage specimens.

(3) Lectins in the cell cycle of normal cells: Noonon and Burger (1973) and Noonan et al. (1973) made the interesting observation that even among normal adult cells that are in general not agglutinated by a lectin there is always a small proportion that are. They identified the agglutinated cells as being those in the process of mitosis, and so they concluded that during mitosis the surface of normal cells is to some extent similar to that of transformed cells. Shoham and Sachs (1974) reported that Con A agglutinability increases during mitosis or normal cells but decreases during mitosis of transformed cells, although the cells bind similar amounts of \(^3\)H-Con A. Increased agglutinability of normal mitotic cells suggests that the surface of normal cells in mitosis bear an intriguing similarity to the transformed cell surfaces. For instance, Glick and Buck (1973) found that
the set of fucose-containing glycopeptides solubilized by trypsin
treatment of mitotic BHK cells more closely resembled the set of
glycopeptides obtained from transformed cells than the set obtained
from interphase normal cells. Also, a study (Garrido, 1975) of Con A
and WGA receptor mobility in CHO cells revealed that, whereas these
receptors are relatively immobile during interphase, they display
the same high degree of lateral mobility during mitosis that one finds
for these receptors in transformed cells.

(4) Lectins in malignant cells: No other property of lectins has
contributed to their importance in biological research as much as their
ability to preferentially agglutinate malignant cells. As often happens,
this property was discovered by chance. The discovery was reported in
1963 by Aub et al., who found that, when a crude preparation of wheat
germ lipase was added to suspensions of isolated mouse lymphoma or
ascites tumour cells, the cells agglutinated, while normal, untrans-
formed cells remained dispersed. The properties of the agglutination
reaction suggested that the active factor in producing aggregation was
not the lipase itself, but a contaminant in the preparation. This
contaminant was later purified and called WGA (Burger and Goldberg,
1967).

In the relatively short period since these original observations
were made, many lectins have been examined and found to agglutinate
selectively the transformed but not the normal cells from a wide variety
of sources. Although most tumours or transformed cells are more readily
agglutinable than their normal or untransformed counterparts, there are
some interesting exclusions from this generalisation. The exclusive
literature pertaining to this field and the wealth of information that
has accumulated, have been the subject of several reviews (Nicolson,
These authors have also examined the mechanism of the complex process of lectin agglutination which appears to be dependent upon a variety of different biological, biochemical and physical parameters. The most recent investigations have lent support to the view that cytoskeletal systems of cells are involved in agglutination.

Agglutination studies are usually performed with cell suspensions mixed with lectins and the results observed on microscope slides. There are variations of this method, such as the hanging drop method of cell agglutination in which small volumes of cells in lectin solutions are mixed, and then a drop examined in an inverted microscope.

Many cells grow in monolayers and are not readily suspended in neutral buffers without trypsin or EDTA treatments. These treatments can modify cell surface characteristics, such as unmasking of cryptic lectin sites. These potential problems and possible cell injury by isolation procedures can be avoided if cell monolayers can be treated in situ with the lectin and this was made possible by the technique devised by Furmanski et al. (1972). They described a haemadsorption technique in which lectin-treated monolayers were overlaid with a suspension of erythrocytes. After a suitable incubation, the erythrocyte suspension was removed and the cell-adherent erythrocytes were counted. Quantitation of the adherent cells is also possible by radio-isotope leballing.

At the level of the light microscope, the distribution of lectin binding sites on individual cells can be observed by autoradiography or fluorescence microscopy. Localization of lectin-binding sites at the high resolution of the EM can be achieved by several procedures (Nicolson, 1980). The most commonly used markers of lectins in transmission electron microscopy has been ferritin, horseradish peroxidase...
and haemocyanin. Lectins tagged with radioisotopes have also been visualized in conjunction with autoradiography at the TEM level. In more recent years, markers and labelling techniques have been developed for visualization of lectin-binding sites by scanning electron microscopy. These techniques and their applications have been described by Brown and Revel (1978), Nicolson (1978) and Molday and Maher (1980). Labelling procedures for SEM observations of cell surfaces are similar to those previously developed for fluorescent microscopy and TEM (see review by De Petris, 1978). The procedures are either direct or indirect. In the former, cells are labelled directly with lectin-marker conjugates in a single step and in the latter, cells are labelled first with the lectin and then subsequently markers are attached to the cell-surface-bound lectins through high affinity specific interactions. In the indirect lectin method, Con A binding sites have been commonly labelled with haemocyanin in a two-step procedure as originally devised by Smith and Revel (1972). Cells are first labelled with Con A, washed in buffer and then incubated with haemocyanin. Since Con A is polyvalent, it can bind to the glycoproteins on the cell surface as well as the glycoprotein haemocyanin.

OBSERVATIONS

Cell surface changes in human papillary tumour urothelium as revealed by lectin-mediated RBC adsorption

In the method adopted for Con A-mediated RBC adsorption (in which RBCs were adsorbed to Con A-coated urothelium) the degree of RBC adherence to the surface seemed to increase with the concentration of Con A. After a series of preliminary experiments, a suitable
concentration was found to be 500 μg/ml and this was subsequently employed for all the lectin experiments reported in this study with RBCs. As mentioned earlier in "Material and Methods", the incubations were carried out at 37°C. The specificity of Con A-coated cells to adsorb RBCs in the present experiments was tested with samples of papillary tumour urothelium. Following incubation with a mixture of Con A and 50 mM each of α-methyl-D-glucopyranoside and α-methyl-D-mannopyranoside, the ability of the urothelial cells to adsorb RBCs was completely abolished. The hapten inhibitors therefore, clearly prevented binding of Con A to the cell surface. The specificity of the other lectins used was also confirmed likewise using appropriate hapten inhibitors.

The large scale adsorption of RBCs to the surface of grade I papillary tumour urothelia, following the use of various lectins, are shown in the representative micrographs presented (Figs. 175, 176 and 177). In these preparations, the RBCs were found in different morphological forms such as discocytes, echinocytes and elliptocytes. The discocytes and echinocytes were common in Con A- and WGA-coated preparations (Figs. 175 and 176) but in preparations using PHA (Fig. 177), there was a preponderance of elliptocytes. It is already known that erythrocyte morphology can be altered by lectins and descriptions of the altered forms have also been provided (Lovriew and Anderson, 1980). It is also believed that the different morphologies which are reversible do not affect the sites of the oligosaccharides of the erythrocyte membrane. Text Fig. 18 and Table 18 show the increase in RBC adsorption on papillary tumour urothelium compared with that on normal aged urothelium. Examination of preparations such as that presented in Figs. 175-177 also showed that most of the RBCs were
present as clusters rather than individual entities. The aggregated RBCs made it somewhat difficult to visualise the surface topography of the underlying cells. With PWM, the number of RBCs adsorbed was very low, and in addition, there were no clusters as observed in experiments with the other lectins.

Cell surface changes in normal human urothelia of different ages as revealed by lectin-mediated RBC adsorption.

Text Fig. 17 and Table 17 give the changes in the mean number of RBCs that were present on the surface of urothelia obtained from the young adults and the aged. The urothelia were coated with different lectins as described earlier, and Figs. 171 and 172 show the RBCs present on urothelia from young adult coated with Con A and WGA respectively. A careful examination of such preparations showed that the few RBCs present were located most often in the gaps that appeared between cells when the tissue was stretched for SEM.

The number of RBCs present on aged urothelia was higher with three of the four lectins used in this study: the exception was PWM (Text Fig. 17 and Table 17). In these preparations (Figs. 173 and 174) also, there were indications of trapped RBCs but some were clearly found on cell surfaces away from the gaps between cells.

In the limited number of preparations that were made, there was also a suggestion that the RBCs adsorptions in the aged urothelia occurred more on the surface of large cells rather than the small ones. The large superficial cells, it may be recalled, are the cells lining the lumen in the thick region of the aged urothelium.
Cell surface changes of Con A-binding in human papillary tumour urothelia as revealed by haemocyanin marker.

The urothelia were treated sequentially with Con A at a concentration of 250 μg/ml and haemocyanin at a concentration of 1 mg/ml. The incubations were carried out at 37°C, as described in "Material and Methods". Control incubation in Con A in the presence of competitive inhibitors of Con A binding as described before, served to demonstrate the specificity of Con A-haemocyanin labelling. Under these conditions there were almost no haemocyanin molecules on the surface of the urothelial cells. The haemocyanin molecule is cylindrical in shape, measuring approximately 35 nm by 50 nm, and when viewed from the end, appear somewhat spherical.

The scanning electron micrograph of a grade I tumour urothelium presented in Fig. 180 shows the variability of the surface features in the tissue. Although the occurrence of microvilli made it difficult to identify haemocyanin molecules unambiguously, it was possible to make out that there were fewer haemocyanin clusters on cells with relatively smooth surfaces and few microvilli, and many more clusters on surfaces presenting more microvilli. In grade II where the cells were more profusely covered with microvilli (Fig. 181) clusters of haemocyanin molecules were even more numerous. When a multivalent lectin such as Con A reacts with carbohydrate groups on the cell surface the intermolecular cross-linking that occurs (Leon, 1975) probably accounts for the clusters mentioned. In view of the topography of the cell surfaces, the limited number of preparations available and the occurrence of clusters no attempt was made to quantify the haemocyanin molecules present and to determine the possible number of cell surface receptors. Moreover, a great deal
Table (17) The mean number of RBCs/100 substrate cells of young and old normal human urothelia.

<table>
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<th>Young</th>
<th>Old</th>
<th>Young</th>
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<th>Young</th>
<th>Old</th>
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<tbody>
<tr>
<td>Con A</td>
<td>4.33±1.221</td>
<td>43.80±8.609</td>
<td>2.64±1.510</td>
<td>24.34±5.198</td>
<td>12.85±4.861</td>
<td>41.43±10.753</td>
<td>2.80±1.395</td>
<td>5.10±1.623</td>
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<tr>
<td>WGA</td>
<td></td>
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<td>PWM</td>
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^P<0.001^P<0.001 J^P<0.001 J^P<0.005

Table (18) The mean number of RBCs/100 substrate cells of old, normal and grade I papillary carcinoma.

<table>
<thead>
<tr>
<th></th>
<th>Old</th>
<th>Tumour</th>
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<th>Old</th>
<th>Tumour</th>
<th>Old</th>
<th>Tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>43.80±8.609</td>
<td>139.90±19.579</td>
<td>24.34±5.198</td>
<td>118.90±7.518</td>
<td>41.43±10.753</td>
<td>160.64±20.251</td>
<td>5.10±1.623</td>
<td>22.85±4.568</td>
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<tr>
<td>WGA</td>
<td></td>
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^P<0.001^P<0.001^P<0.001^P<0.005
Text Fig.(17) The mean number of RBCs/100 substrate cells of young and old urothelia.

Text Fig.(18) The mean number of RBCs/100 substrate cells of old normal urothelium and grade I papillary carcinoma.
of evidence has accumulated in the literature which argues against using haemocyanin molecule counts to estimate receptor numbers (Cf. Molday and Maher, 1980). The greatest dimension of Con A molecule is 9 nm and therefore one haemocyanin molecule (35 × 50 nm) could cover several closely juxtaposed Con A molecules and/or binding sites.

Age related changes of Con A-binding in normal human urothelia as revealed by haemocyanin marker.

The surface features of young urothelium did not present any difficulty in detecting haemocyanin molecules. In preparations such as that shown in Fig. 178, it was obvious that there were very few randomly dispersed haemocyanin molecules on the surface of the cells. On the other hand, a markedly greater number of the molecules were apparent on the surface of the urothelium of the elderly subjects (Fig. 179). The distribution of the molecules was not quite even because, in places, multiple aggregates or clusters of haemocyanin were evident. Single molecules, however, were also present between the aggregates.

DISCUSSION

The involvement of the cell surface in several functions of the cell has been increasingly recognised in recent years. Also well recognised is the fact that the cell surface properties are determined by the chemical nature and conformation of the sugar components of the cell surface in the form of glycoproteins and glycolipids. Many of the plasma membrane proteins, especially the integral proteins, are glycoproteins whose carbohydrate-rich proteins – oligosaccharide chains – extend outward from the outer surface of the
membrane. The cell surface glycoconjugates are involved in cell-to-cell interactions and in determining the physico-chemical properties, homeostatic functions, receptor functions and antigenic properties of the cell. Lectins, which are proteins extracted from various plant and animal sources, have high affinities for specific sugar residues and hence have been extensively used as molecular probes for investigating the nature of sugar residues and their distribution on cell surfaces. As mentioned earlier, lectins have been used as probes for investigating changes in cell surfaces during embryonic growth, differentiation, and morphogenesis in vitro cell ageing, changes in membrane structure and organisation after infection by oncogenic and non-oncogenic viruses, and after neoplastic transformation by various agents. In the present study, an attempt was made with four lectins possessing different saccharide specificities as probes to detect possible cell surface changes in the urothelium of human bladder related to ageing and neoplasia. There are no previous reports of such an approach to examine these aspects on the surface of human urothelium.

Electron microscope studies have shown (as described elsewhere in this thesis) that the luminal plasma membrane does undergo structural changes with age as indicated by decreasing proportion of asymmetric membrane plaques, and increasing flexibility of the surface with the tendency to form smooth contours and microvilli-like structures. The surface cells lining the lumen of the bladder in the elderly are also not so well differentiated as in the younger age groups. Age related changes at the cell surface are hence likely. The cell surface changes looked for were the density of binding of lectins to the surface and the pattern or distribution of binding
sites. Data from the lectin-mediated RBC adsorption experiments provide an indication of the presence or absence of specific receptors and also their mobility. In the preparations with normal young urothelia, very few RBCs were present and these were located almost exclusively in the artificial gaps between the stretched cells rather than the surface of cells. If, as would seem then, these are instances of non-specific trapping of RBCs rather than adsorption to bond lectin, one may conclude that carbohydrate residues, specific for the lectins employed in this limited study, are present in very low amounts or are practically absent on urothelial surface of young subjects. In the urothelia of aged subjects, larger numbers of RBCs were found following coating with Con A, WGA and PHA and the RBCs were clearly located on the surface of cells rather than in gaps between cells. This distribution is more likely to represent RBCs adsorption, although some trapped RBCs were present as explained before and to about the same extent as in the young urothelia. Indications from this preliminary set of experiments, therefore, are that the surface of aged urothelia bind more lectins than that of the young tissue. In future experiments, it should be possible to check if the degree of binding can be accentuated by reacting the aged urothelium simultaneously with Con A and WGA, since the sites for the two lectins may be spatially distinct. Prior to such experiments, more preliminary work would be necessary to standardize the degree of stretching of the tissue before the incubation and to devise effective washing procedures to remove unadsorbed markers.

The surface distribution of Con A examined by haemocyanin adsorption points to a substantial increase in the amount of lectin binding in the aged urothelium compared with the young urothelium. The markedly higher degree of labelling with haemocyanin compared
to that with RBCs may be due to the small size of the haemocyanin molecules. This would enable much greater accessibility of the marker for the binding sites and probably also less chance of the marker molecule being sheared off during washing of the sample in the course of preparation. The surface of the aged urothelium showed not only a greater number of haemocyanin molecules but also aggregates of the molecules as though there was clustering of receptor sites. This would be interesting since fixation of cells prior to lectin coating is known to restrict lateral mobility and hence redistribution of the receptor sites (Nicolson, 1974, 1976, 1980; Brown and Hunt, 1978; Molday and Maher, 1980). Prior fixation, however, does not affect the binding of lectins to receptors and glutaraldehyde-fixed cells are often used to map the inherent distribution of antigens and receptors on cells. The apparent clustering mentioned above may merely reflect increased local densities of receptors rather than rearrangement facilitated by receptor movement. Further, it may also be pointed out that enhanced mobility and clustering of receptors are not invariably associated with agglutination and transformation as pointed out by several workers (Nicolson, 1976; 1980; Brown and Hunt, 1978). In human fibroblasts, Aizawa et al. (1980b) found a close similarity in cell surface properties between senescent cells and transformed cells which led them to question the view that high lectin-mediated agglutinability and RBC adsorption are intrinsic to neoplastic transformation.

A discussion of cell surface is complicated because it may refer to the basic plasma membrane, the membrane associated glycocalyx, or both. The glycocalyx contains carbohydrates and proteins, including glycosaminoglycans (Kraemer, 1971; Vogel and Kelley, 1977). The glycocalyx probably does not bind directly to the plasma membrane
but is only apposed to the cell surface as is the case with secreted material. Since the glycocalyx as well as the plasma membrane bear oligosaccharides, and hence lectin-binding sites, it is operationally difficult to distinguish between the roles of these entities in relation to various cell surface properties and phenomena. Accounts of cell surfaces in the literature is also confusing because of the semantic problem. An increase in the thickness of the glycocalyx has been demonstrated in the aged urothelium and this may account for the increased binding of Con A and the other lectins used in the present study. Another explanation of the increased lectin binding may be in qualitative differences in the glycocalyx material of the aged urothelial cells compared with that of the young urothelium. On the other hand, if the glycocalyx does not prevent accessibility to oligosaccharide borne on the integral membrane proteins, the binding of lectins observed on the surface of the aged tissue may mean the appearance of new plasma membrane oligosaccharides which are capable of binding to the lectins used, namely Con A, WGA and PHA but not PWM. In this context, it will be of interest in the future to compare the lectin binding characteristics of foetal urothelia by the methods adopted in the present study.

The RBC and haemocyanin adsorption assays show very pronounced binding of Con A, WGA and PHA (but not PWM) to the urothelial surface of low grade papillary tumours as compared with normal urothelia. The surfaces of the tumour urothelia possess increasing numbers and increasing pleomorphism of microvilli, as described earlier, and the consequent amplification of the cell surface could account for the increase in the number of lectin-binding sites. It is even possible that increasing numbers of lectin receptors are concentrated on the
microvilli. In addition, transmission electron microscope studies have demonstrated a progressive increase in the thickness of the glycocalyx of the papillary carcinomas grade I and II. As explained earlier, the glycocalyces in these tumour urothelia were visible in routine preparations unlike those in normal urothelia where it could be visualised only by the use of a special stain. It is therefore possible that there are qualitative as well as quantitative differences between the glycocalyx material of the normal and the neoplastic urothelia. Other staining methods, such as colloidal iron and periodic acid, apart from ruthenium red, may provide ultrastructural indications of differences in the composition of the glycocalyx. For the present, one may tentatively conclude that the increase in the thickness of the glycocalyx contributes to the increase in receptor molecules of Con A and the other lectins used, although there are several reports (reviewed by Hynes, 1976) that the composition of the glycocalyx layers may also differ in normal and transformed cells. Examples of instances in the literature where the thickness of the glycocalyx and/or the rate of synthesis of the glycocalyx increased with transformation have been cited elsewhere in this thesis and are not repeated here. General changes in the overall composition of plasma membrane glycoproteins and glycolipids have been found in a variety of tumours and transformed cells (Hynes, 1976; Nicolson, 1976). Such changes may also apply to the present material and these remain to be further investigated using isolated membrane fractions. The possible contribution of both the plasma membrane and the glycocalyx to lectin binding capacity and the presence of microvilli make it difficult in the present case to consider the question of agglutination
in the tumour urothelia. The vastly increased densities of lectin receptors in the tumour cells compared with normal bladder urothelia is however evident.

As a result of the present investigations of cell surfaces with lectins, one may venture to suggest a rapid and easy way to detect early neoplasia in human bladder urothelium. If suspected biopsies are pinned to a wax-coated Petri dish in a manner analogous to tissue preparation for scanning electron microscopy, the tissue can be sequentially incubated with Con A and RBC suspension as described in "Material and Methods". Trial runs of this procedure have shown that the large-scale adsorption of RBCs on the cells can be easily observed in the light microscope and distinguished from the practically negative reaction with normal healthy urothelia. The procedure, adapted from the original monolayer technique of Furmanski et al. (1972), has similarities to agglutination assays of isolated cells described in numerous tumour cell systems. Agglutinability of isolated bladder cells of rat urothelium with lectins, as carried out by Kakizoo et al. (1979), is neither desirable nor feasible in the case of human urothelium. Their method of isolation by sonication damaged most cells as indicated by trypan blue exclusion test. Alternative methods of isolation using proteolytic enzymes have several well known disadvantages for study of lectin mediated receptors (Nicolson, 1974; Rapin and Burger, 1974). In addition, there are reports (Follett and Goldman, 1970; Willingham and Pastan, 1975) that trypsin treatment of mouse 3T3 cells increases the number of microvilli. In any case, the size of human bladder biopsies that are usually obtainable preclude cell isolation procedures. With further tests and standardization of procedures, the above method of labelling intact tissue with lectins
such as Con A may be developed as a rapid and reliable histopathological means of diagnosing early papillary carcinoma.
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