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Observations on the Ultrastructure of Human Urothelium: The Response of Normal Bladder of Elderly Subjects to Hyperthermia

J. Jacob[1], J. R. Hindmarsh[2*], C. M. Ludgate[2*] and G. D. Chisholm[2]

[1] Department of Genetics, University of Edinburgh, Edinburgh, U. K.
[2] Department of Surgery/Urology, Western General Hospital, Edinburgh, U.K.

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Summary. An electron microscopic study of normal bladder urothelium of elderly subjects treated by hyperthermic perfusions has shown that the tissue responds, sooner or later, in every instance by desquamation. There is no evidence of cell death prior to desquamation although various organelles undergo structural alterations. Mitochondria are especially prone to suffer varying degrees of damage. A short heat shock has revealed differences in the initial response of the thick and thin regions of bladder urothelium known to occur in elderly subjects. After a long, fractionated treatment, regeneration is evident within 3 days of the end of treatment, and follow-up biopsies have revealed a hyperplastic urothelium within 10 to 12 weeks. The constituent cells show signs of cytodifferentiation at this time but it remains unknown when an ultrastructurally normal urothelium with characteristic cell layers will be restored. The various treatments in this study suggest that the stem cells in the epithelium are unaffected by the levels of hyperthermia employed and that their unimpaired proliferative capacity ensures regeneration of the urothelium.

Key words: Human, Bladder urothelium, Electron microscopy, Hyperthermia.

Introduction

In recent years, there have been several reviews on the susceptibility of tumour cells to heat, and an associated revival of interest in the use of hyperthermia in the treatment of cancer [1, 5, 9, 19, 23]. Attempts have also been made to treat transitional cell carcinoma of the urinary bladder by local hyperthermia [3, 8, 16, 17] but there is little information in these accounts on the effects of elevated temperatures on the intervening areas of normal epithelium (urothelium) lining the bladder. During hyperthermic treatment of bladder carcinoma, the normal urothelium is unavoidably subjected to heat, and this report describes its in vivo response to the treatment. Some of the findings of this report have been published in preliminary communications [13, 14]. The ultrastructure of the normal urothelium of elderly subjects has been studied in detail earlier [12] and that account will serve as the basis for comparison with the present observations.

Materials and Methods

Biopsies were obtained from ten patients who were selected for treatment by hyperthermic perfusions at 44 °C. All were elderly subjects with different grades/stages of recurrent bladder tumours occurring at various times after radical radiotherapy. The interval between radiotherapy and hyperthermia in all cases was at least 6 months. The selection of patients was difficult because it was impossible to find individuals with identical medical history and course of treatment. Some of them have had diathermy and others resection and chemotherapy, before radiotherapy, while yet others had had all these treatments. Therefore, as in most investigations involving human biopsies [4] the collection of material and the experimental design were a compromise between what is ideally required and what is ethically acceptable and clinically expedient. In all, 16 biopsies were collected, some immediately after treatment and others at various intervals subsequently, but the final analysis was restricted to nine biopsies from six patients.

The method of hyperthermic perfusion of bladder has been described previously [16]. The biopsies were collected with Storz forceps, fixed and processed for electron microscopy by routine procedures described before [12]. For autoradiographic monitoring of cellular function as indicated by macromolecular syntheses, small portions of biopsies were cultured in vitro for 2 h at 37 °C in medium-199 enriched with 10% fetal calf serum and added radioactive tracer: 100 μCi/ml 5-^{3}H-uridine, 29 Ci/mmole, or 100 μCi/ml L-4, 5-^{3}H-lysine, 18 Ci/mmole (Radiochemical Centre, Amersham). The material was then embedded in epoxy resin after appropriate fixation – washing schedules [11] designed to avoid artefacts due to retention of free label. Sections were cut 1 μm thick for dipping in Ilford L4 nuclear

* Present address: Mr. J. R. Hindmarsh, Institute of Urology, London WC2H 8JE, England; Mr. C. M. Ludgate, Cancer Control Agency of British Columbia, Vancouver, Canada

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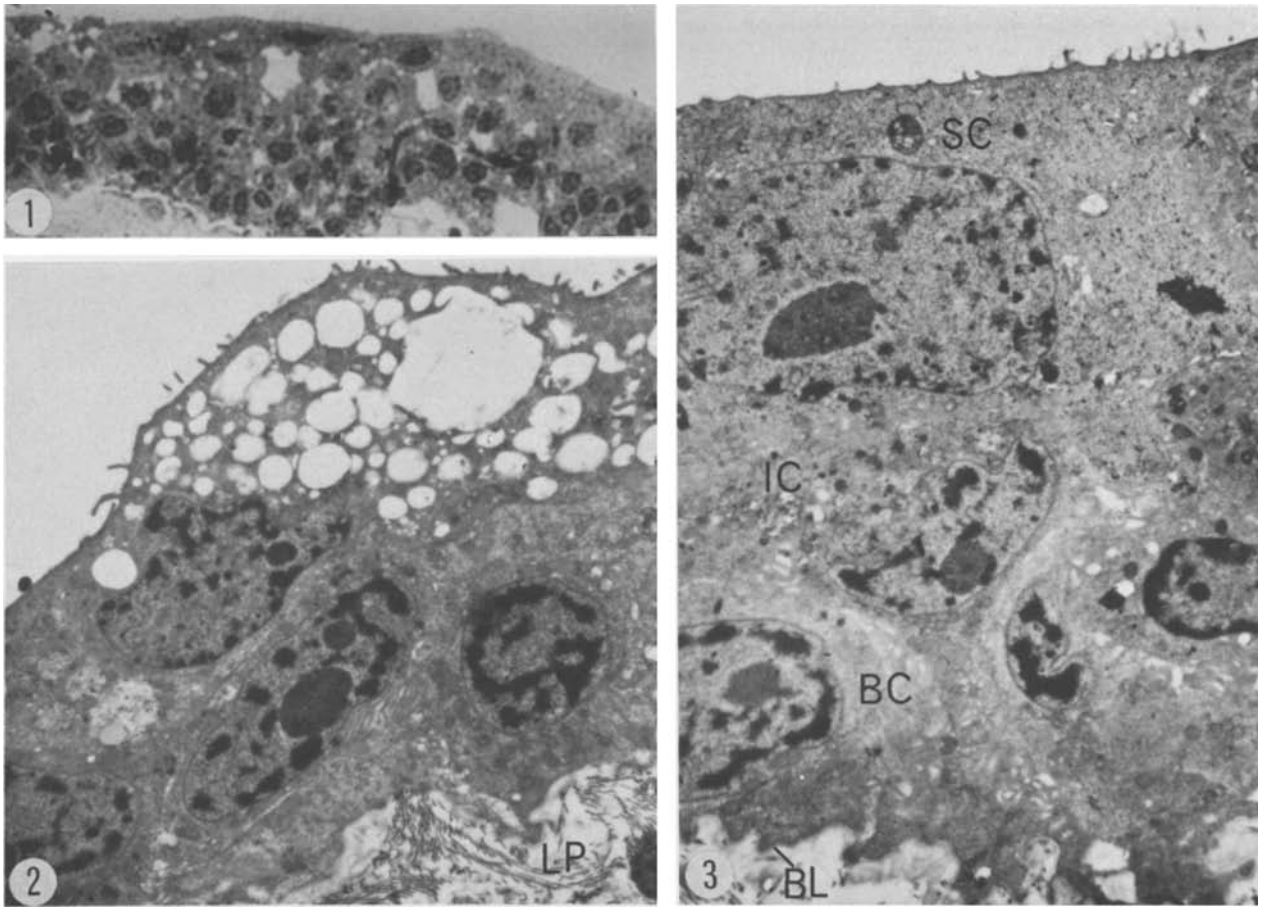


Plate I. Micrographs showing the general appearance of urothelium 4 days after 2 h heat treatment

Fig. 1. A light micrograph of a stretch of urothelium consisting of several layers of undifferentiated cells. Basic Fuchsin strain (x 460)

Fig. 2. A low power electron micrograph showing two layers of undifferentiated cells. A highly vacuolated cell that will soon be lost is visible at the luminal surface. *LP*, lamina propria (x 5,100)

Fig. 3. Low power micrograph showing a region where a polarised organisation of cells persists with a large superficial cell (SC). *IC*, intermediate cell; *BC*, basal cell; *BL*, basal lamina (x 5,100)

emulsion, and the autoradiographs processed after 4 weeks exposure were finally stained in basic Fuchsin before examination in a light microscope.

Results

Short Heat Treatments

Patients treated by hyperthermic perfusion at 44 °C for 1 h or 2 h in order to control bleeding from tumours provided the material for examining the early heat effects. Although gross haematuria was arrested by this treatment, electron micrographs of tissue examined immediately after 1 h of perfusion showed no marked changes when compared with earlier observations [12] on normal urothelium of elderly subjects. This is in aggrement with the findings of Ludgate et al. [16], who had pointed out that, at cytstoscopy after 1 h perfusion, the normal mucosa appeared healthy. At the beginning of hyperthermic treatments therefore, the normal urothelium appeared to be free of cytological changes arising from previous treatments including radiotherapy 6 or more months ago.

Some changes were, however, observed in tissues obtained 4 days after a 2 h treatment. In some regions, the urothelium consisted of small undifferentiated cells forming as many as five or as few as two layers (Plate I, Figs. 1 and 2). There was also evidence of desquamation along these regions. In the remaining regions, the urothelium appeared to be normal with three to four layers of cells of which the superficial ones were the largest and most differentiated (Fig. 3). Desquamation was not noted along these stretches. The appearance of the nuclei of superficial cells was not manifestly different from that in normal untreated tissue but the nuclei of intermediate and basal cells showed a progressively greater proportion than usual of their chromatin in a con-

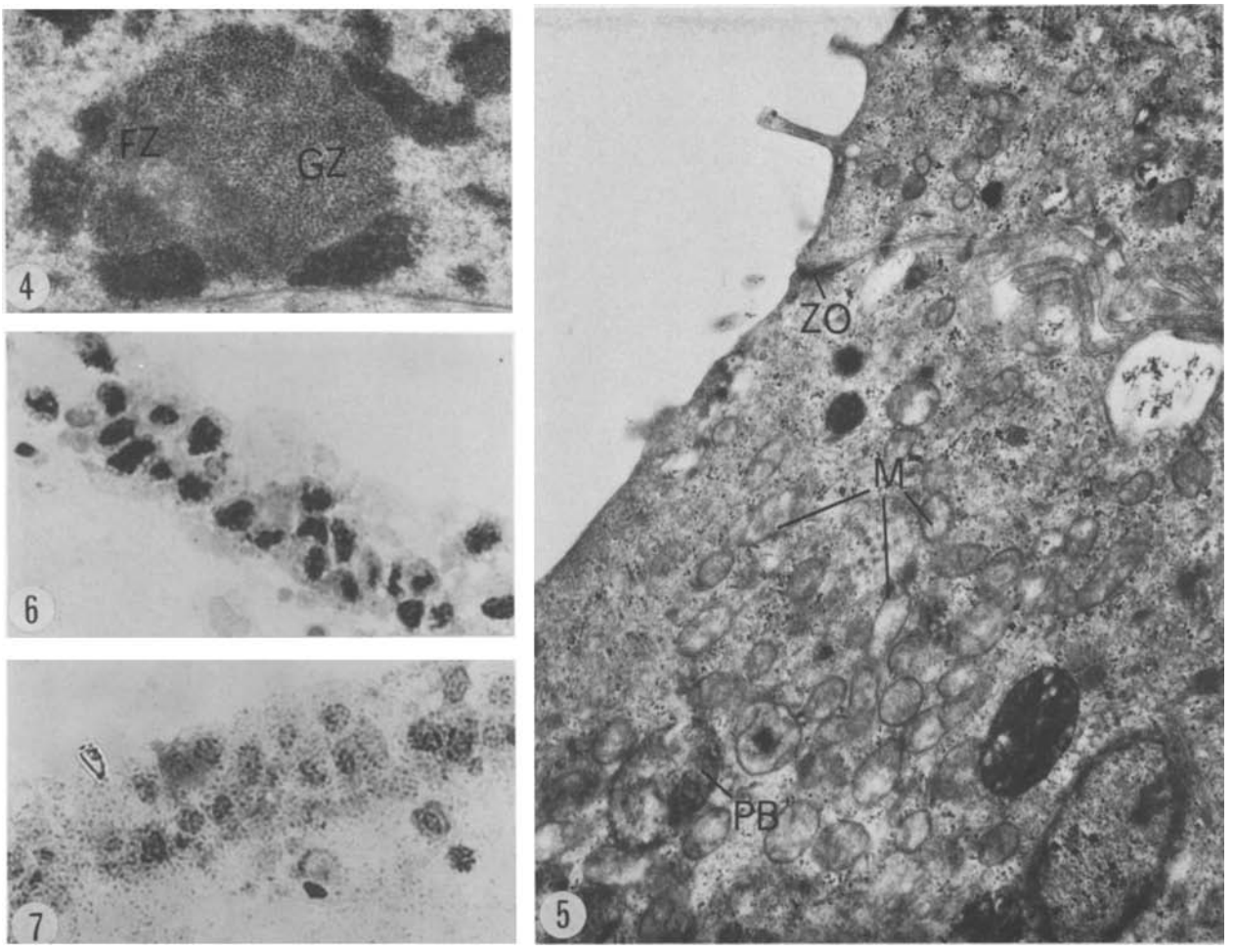


Plate II. Micrographs of urothelium 4 days after 2 h heat treatment

Fig. 4. A high power electron micrograph to show segregated nucleolus in a basal cell of the differentiated region of urothelium. FZ, fibrillar zone of nucleolus; *GZ*, granular zone of nucleolus (x 25,500)

Fig. 5. Abnormal looking mitochondria *(M)* in a superficial cell of the differentiated region. *ZO*, zonula occludens; *PB*, polymorphic body (x 17,000)

Figs. 6 and 7. Light microscope autoradiographs showing uptake by urothelial cells of ^{3}H-uridine and ^{3}H-lysine respectively (x 410)

densed form. A similar gradation was evident with regard to alterations in the structure of nucleoli. In the superficial cells, nucleoli seemed normal (Fig. 3) but in the basal cells, there was a clear segregation of particulate and fibrillar components into distinct zones (Plate II, Fig. 4). A somewhat intermediate condition prevailed in the intermediate layer.

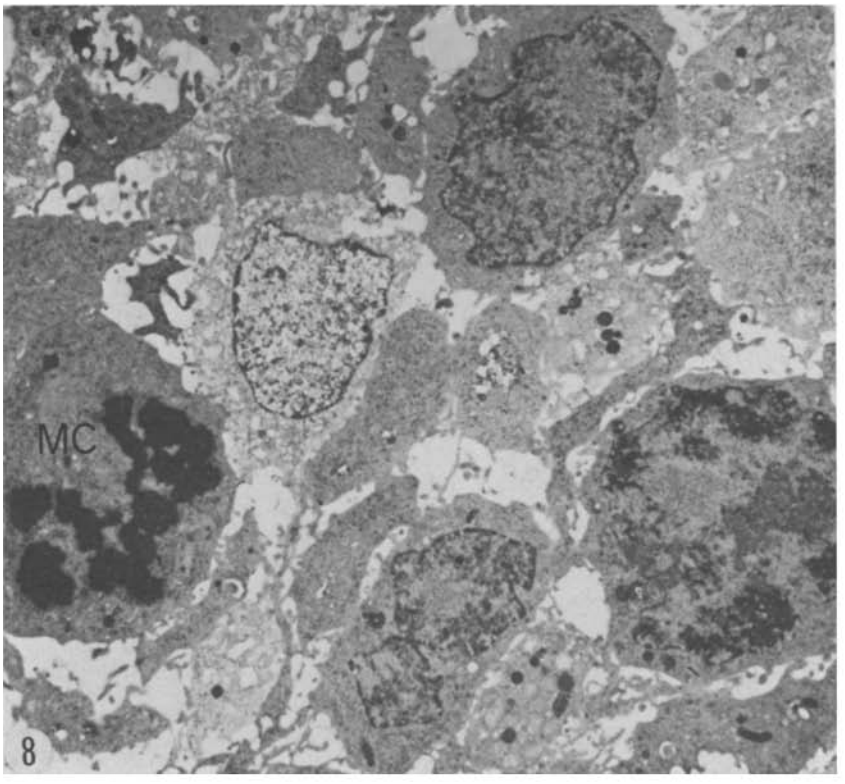
Abnormal-looking mitochondria were found in cells of all regions of the urothelium. A cluster of such mitochondria in a superficial cell is shown in Fig. 5; there were few cristae and their matrices were electron translucent. The Golgi apparatuses seen in the superficial cells were small and contained only a few cisternal profiles, unlike in normal urothelium where large well developed Golgi complexes occur with stacks of cisternae and vesicles of various sizes.

Light microscope autoradiographs prepared by incubating the biopsies in radioactive uridine of lysine as soon as they were collected showed uptake of both the precursors (Figs. 6 and 7).

Short Treatment Plus a Long Fractionated One

This material became available when one of the above patients who was treated for 2 h at 44 °C to stop intractable haemorrhage was given an additional course of fractionated treatment 13 days later, as treatment for recurrent carcinoma. The second treatment lasted 9 days and consisted of 1 h perfusions at 44 °C on 5 alternate days.

Biopsies were taken 2 days after the end of the treatments when the urothelium was found to be more or less uniformly thick with four to six cell layers. Desquamation seemed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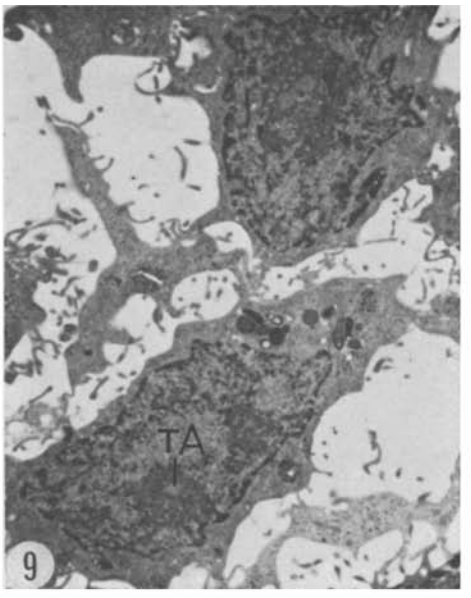
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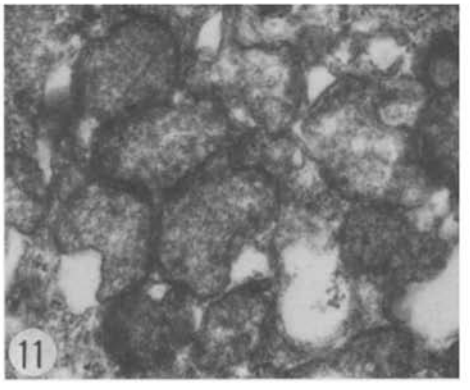
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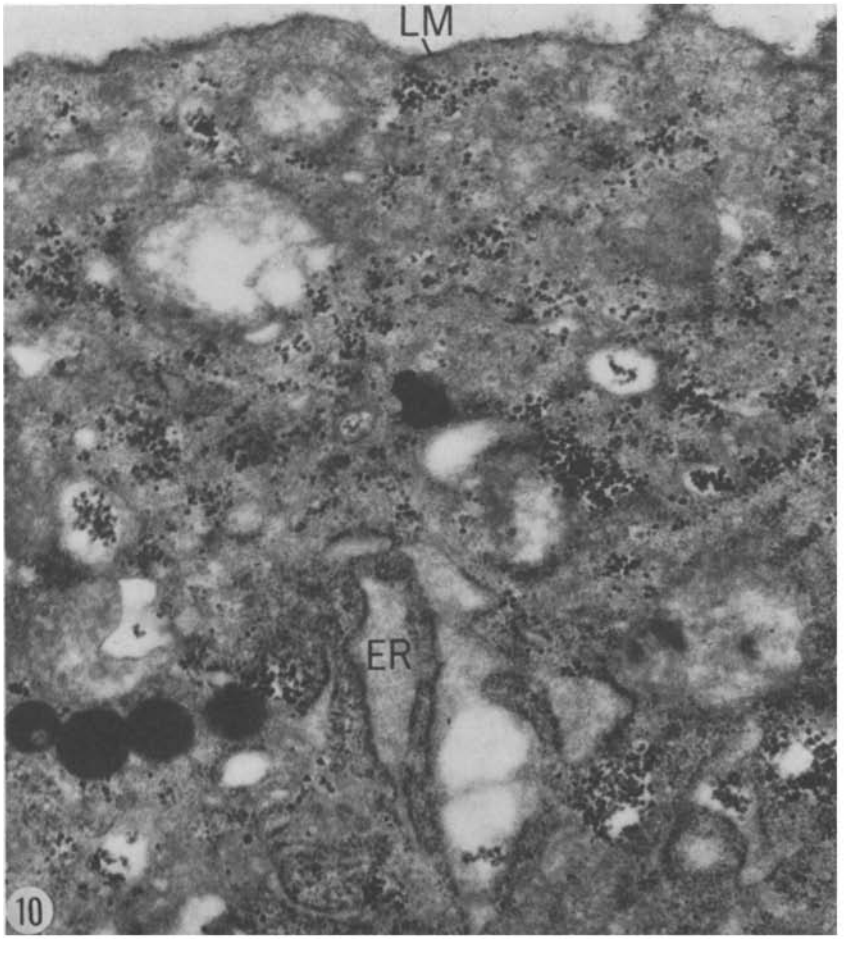
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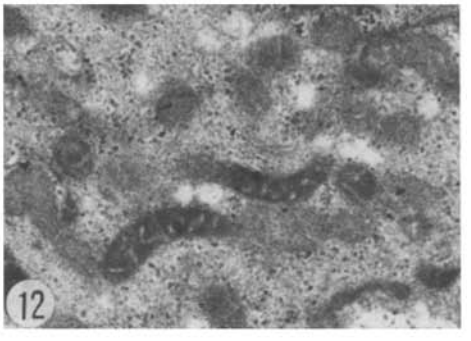
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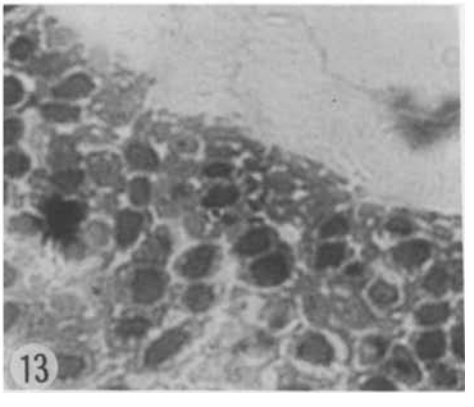
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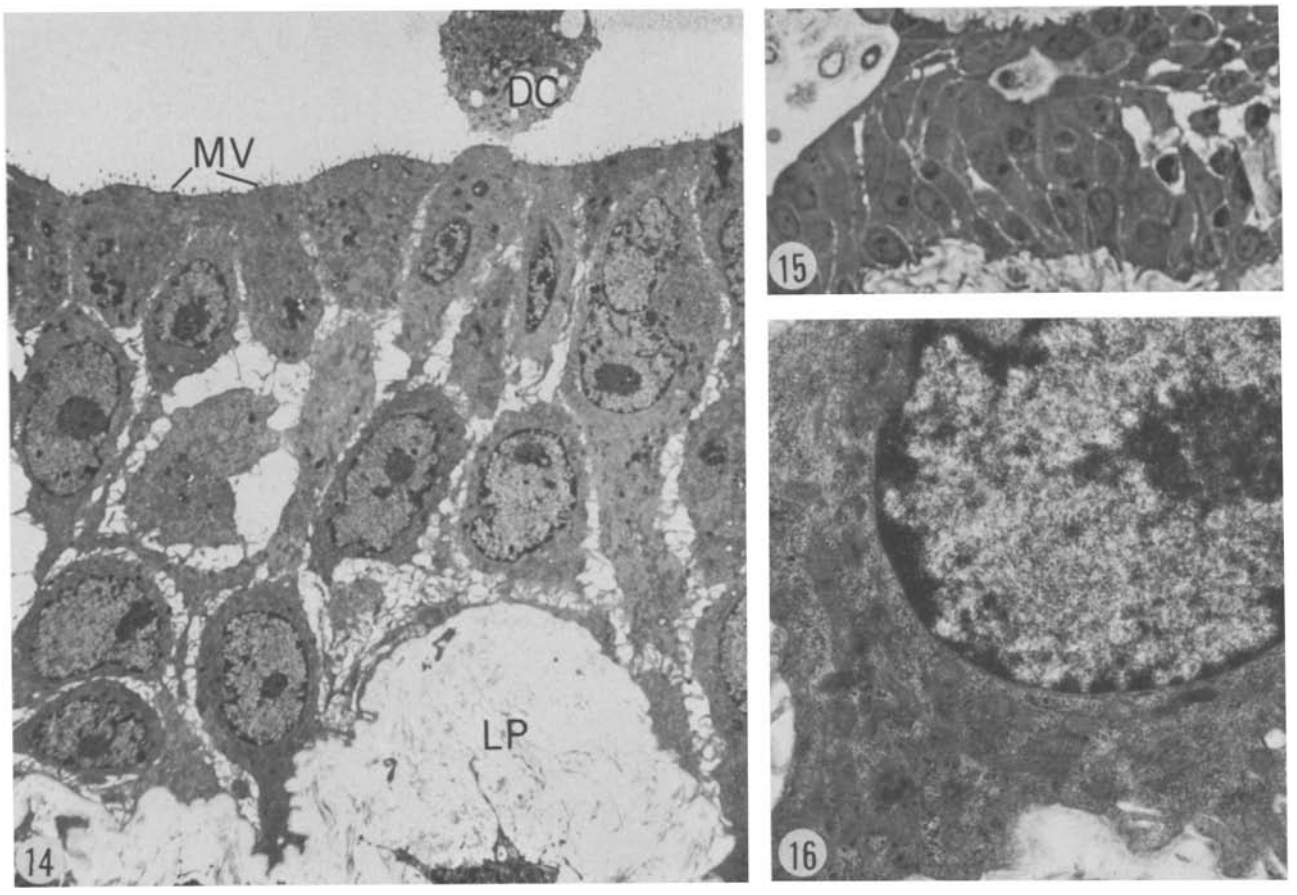


Plate IV. Micrographs of urothelium 3 days after a long fractionated heat treatment

Fig. 14. A low power picture to show the presence of uniformly small cells. *MV*, microvilli; *LP*, lamina propria; *DC*, desquamating cell (x 2,600)

Fig. 15. A light micrograph to show the variable number of cell layers present (x 520)

Fig. 16. High power electron micrograph of a normal looking basal cell (x 12,700)

moderate and the epithelium was infiltrated by connective tissue cells which included polymorphonuclear leukocytes.

An unusual observation was the sporadic occurrence of mitotic cells (Plate III, Fig. 8) but apart from this, the population of epithelial cells was strikingly homogeneous – all being small and undifferentiated. In the nuclei, the chromatin was invariably distributed like a lace made up of both diffuse and condensed chromatin and the nucleoli showed reticulate organisation enclosing translucent areas (Fig. 9). The cytoplasm was of low electron density, contained many vacuoles and was reduced in size to form a ring around the nucleus. The plasma membranes of cells

◀ **Plate III.** Micrographs of urothelium 2 days after a course of short and long treatments

Fig. 8. A low power electron micrograph showing a mitotic cell *(MC)* amidst small, undifferentiated cells (x 5,100)

Fig. 9. A micrograph to show the peculiar distribution of chromatin in cell nuclei. Note also translucent areas *(TA)* within nucleoli (x 5,100)

Fig. 10. A high power micrograph of the periphery of a cell to show indistinct luminal plasma membrane *(PM)*, flocculent cytoplasm and distended endoplasmic reticulum *(ER)* (x 25,500)

Fig. 11. A cluster of swollen mitochondria devoid of any internal organisation (x 25,500)

Fig. 12. Another cluster of abnormal mitochondria with very narrow cristae embedded in electron dense matrix (x 25,500)

Fig. 13. A light microscope autoradiograph to show lack of uptake of ^{3}H-lysine by urothelial cells (x 560)

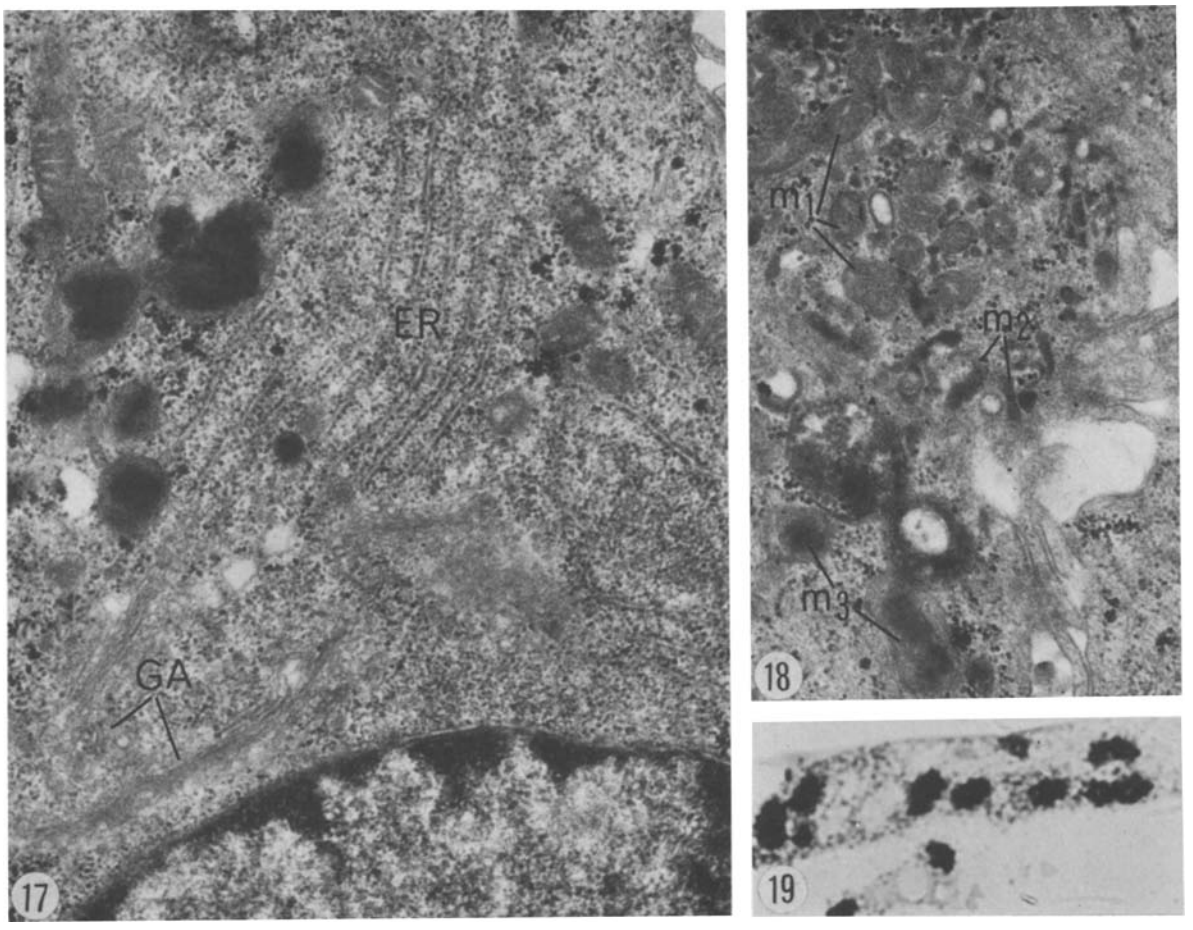


Plate V. High power electron micrographs of urothelial cells 3 days after fractionated treatment

Fig. 17. Golgi apparatus *(AG)* are poorly developed but several profiles of endoplasmic reticulum *(ER)* are visible. Note the occurrence of several kinds of mitochondria as in Fig. 18 (x 25,500)

Fig. 18. Several kinds of mitochondria are visible: m_1, mitochondria of usual size and shape; m_2, highly shrunk entities which probably are degenerating mitochondria; m_3, abnormally large mitochondria with electron dense interior (x 25,500)

Fig. 19. Autoradiograph showing incorporation of radiouridine (x 410)

were indistinct. No organelles could be clearly distinguished in the flocculent-looking cytoplasm. Rarely, what seemed to be distended cisternae of endoplasmic reticulum (Fig. 10) were encountered. As for mitochondria, sometimes whole clusters consisted of swollen and highly irregular forms devoid of any internal organisation (Fig. 11). In other instances, the organelles retained their characteristic overall appearance but the cristae were very narrow and seemed embedded in an electron dense matrix (Fig. 12).

In the lamina propria, several leukocytes were present and there were also morphological indications that pinocytotic vesicle transport was affected in the endothelial cells. Autoradiographs of the biopsy cultured in tritiated uridine or lysine (Fig. 13) failed to reveal any incorporation.

Long Fractionated Treatment

In this case, the bladder was subjected only to a fractionated heat treatment which consisted of five, 1 h perfusions lasting 9 days as already described. Examination of biopsies collected 3 days after the treatment showed a four to six layer thick urothelium consisting of small cells, although regions with fewer cell layers were occasionally noted (Plate IV, Figs. 14 and 15). In both light and electron microscope preparations, desquamating cells were observed frequently.

The ultrastructure of cells in the basal layer (Fig. 16) was, for the most part, similar to that of corresponding cells in normal urothelium except for instances of mitochondria with somewhat electron dense matrices. Although the rest

of the urothelium was also made up of similar small cells, many of them exhibited features that are usually associated with metabolically active cells. The nucleoli were relatively prominent and predominantly granular and much of the nuclear chromatin was in a diffuse state leaving only a very narrow layer of condensed chromatin along the margin of the nuclear envelope. In the cytoplasm, fairly extensive profiles of rough endoplasmic reticulum were present in some of the cells although their Golgi apparatuses were poorly developed (Plate V, Fig. 17). There was no dearth of mitochondria in the cells of the peripheral and middle layers of the urothelium (Fig. 18). Most of them were of the usual size and shape but the matrix was electron dense. A small proportion of mitochondria were larger than usual and possessed dense patches within. Another kind which also constituted a small proportion consisted of dense and greatly shrunk entities (Fig. 18). Autoradiographs of this tissue showed heavy labelling with uridine (Fig. 19) and lysine.

In a biopsy examined 10 weeks after a long fractionated treatment, the urothelium was found to be even thicker with up to eight or nine layers of cells (Plate VI, Fig. 20). The nuclear-cytoplasmic ratio of the cells was now lower than that in the tissue described above 3 days after the treatment. Cell loss from the urothelium appeared to have ceased and there was a high incidence of infiltrating lymphocytes.

The chromatin was on the whole diffuse and nucleoli granular as described above but the nuclei were highly irregular in shape with deep indentations. Further features of interest were in the cytoplasm. Mitochondria now generally appeared normal with well defined cristae and matrices of usual density (Figs. 21–23); there were even some large ones measuring over 6 μm in length. Although large numbers of mitochondria were present, they were seldom seen in clusters as in normal urothelium. Golgi complexes were noted in several cells and unlike in the previous biopsy, they were more extensive and well developed with both cisternal and vesicular components (Fig. 22). Vesicles of similar appearance were also observed in peripheral regions of the cytoplasm close to the luminal plasma membrane (Fig. 21). Another notable feature was the presence in several cells of rough endoplasmic reticulum (Fig. 23) which seemed to be even more extensive than that reported in the most differentiated superficial cells of normal urothelium. The cells exhibiting these features were not arranged in any regular manner; instead they tended to occur in a haphazard manner and in different cell layers.

When tissue from another patient was collected 12 weeks after the fractionated treatment, the urothelium was found to be multilayered, as above, but organelle development was less pronounced.

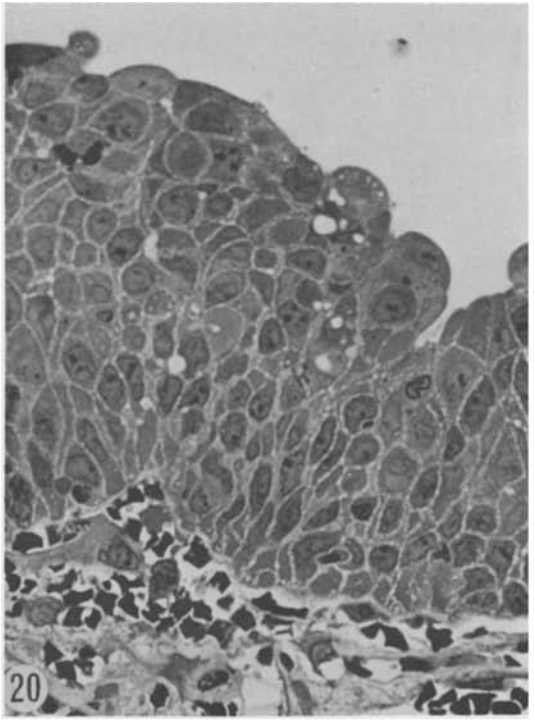
Discussion

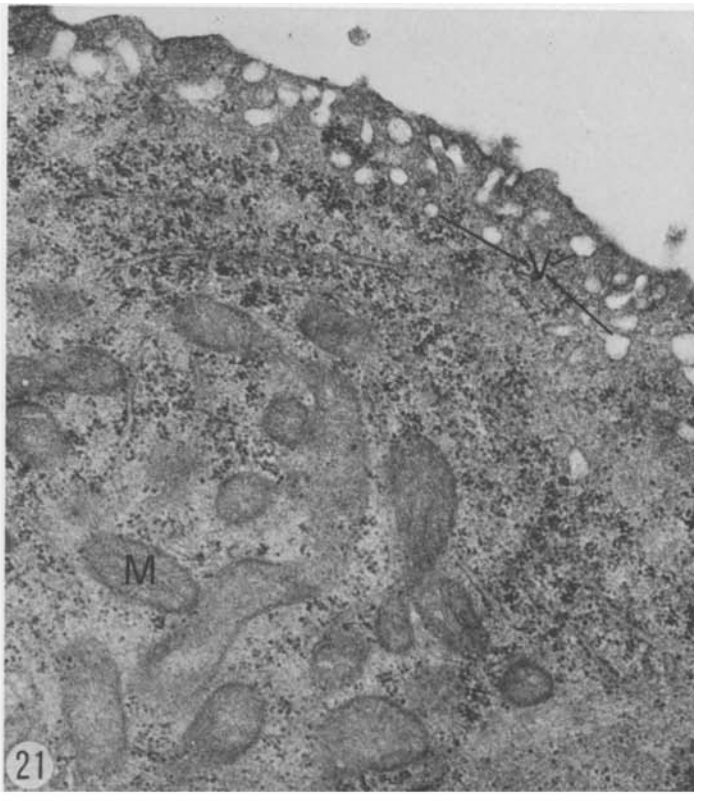
In the present series of biopsies, the earliest heat effects observed were in material obtained 4 days after 2 h perfusion. The effects varied in different regions of the urothelium. Some regions were made up of small undifferentiated cells forming two to five layers; there was also desquamation along these stretches. The occurrence of unusually thin stretches of urothelium consisting of only one or two layers of undifferentiated cells interspersed between thick regions of normally differentiated cells has been previously reported in the bladders of elderly subjects [12]. The present observation would then suggest that the thin stretches are unstable sites where a rapid turnover of cells can be induces by mild trauma such as a brief heat shock. The fact that the undifferentiated cells come to form more layers than the original one or two, further suggests that there is faster cell proliferation than cell loss.

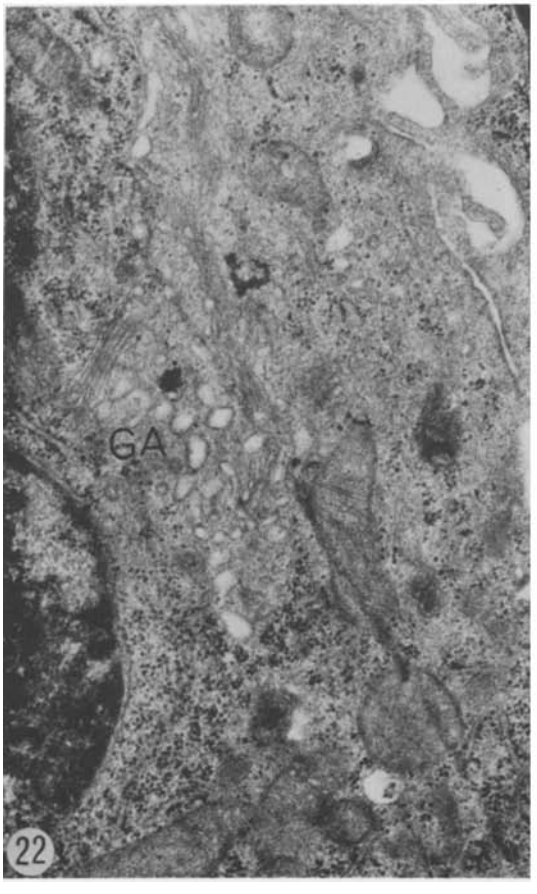
There is no doubt that the 2 h treatment has also affected, in a less dramatic way, the normally differentiated urothelial regions consisting of cells with a pattern of cytodifferentiation progressing from the basal to the superficial layer. The lack of desquamation along these sites may be regarded as an indication that the normally differentiated urothelial regions differ from the undifferentiated regions in their immediate response to heat. Nevertheless, by the 4 th day, there is an increase in the proportion of condensed chromatin or a shift to a higher heterochromatin-to-euchromatin ratio, especially in the intermediate and lower cell layers which might be interpreted as a sign of depressed synthetic activity. Likewise, the nucleolar alteration manifested most clearly in the basal cells as a segregation of structural components into distinct masses, is widely accepted as a phenomenon caused by agents that interfere with the template activity of nucleolar DNA. Nucleolar segregation is generally held to be a response to some kind of structural changes in DNA rather than a consequence of mere inhibition of RNA synthesis [21]. The nuclear and nucleolar alterations in the urothelial cells may therefore be attributed to the effect of heat on the physical state of their chromatin. Our knowledge of the molecular mechanisms involved in the transformation of one state of chromatin to the other is limited. It still remains uncertain whether template-active and template-inactive states differ in organisation at the nucleosomal or supranucleosomal level [6, 24, 25] although it is known that histone H1-DNA or H1-H1 interactions are involved in the maintenance of the highly condensed state [18, 26].

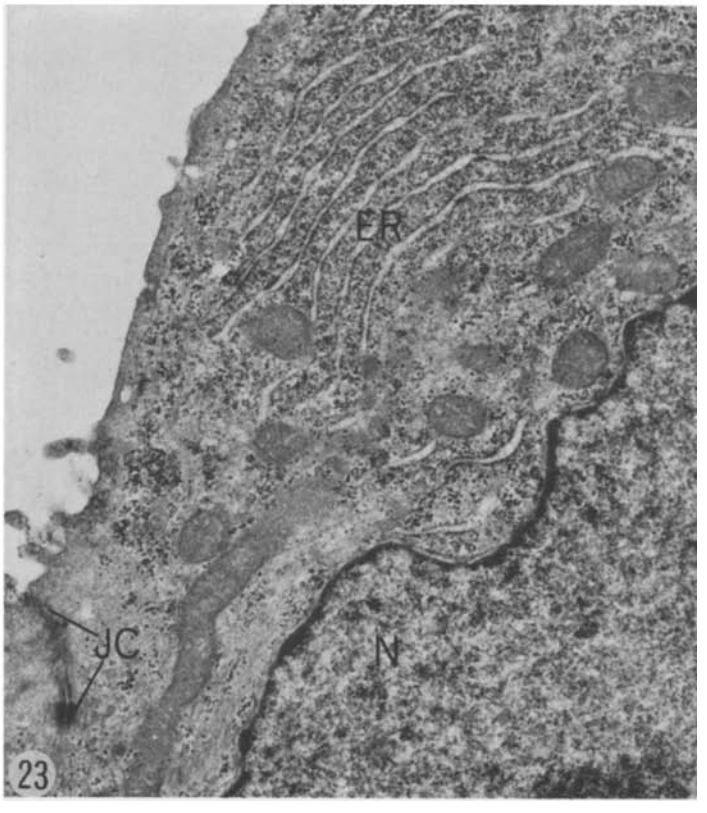
At the ultrastructural level, the other lesions observed 4 days after the brief heat treatment are damaged mitochondria in all cells and decreased Golgi activity in the superficial cells of the differentiated regions of the urothelium. Although these and the other lesions mentioned above indicate functional impairment in these cells, autoradiographs show that they have not yet led to a cessation of macromolecular syntheses.

The state of the urothelium 2 days after the end of a long fractionated treatment which followed 13 days after an initial 2 h treatment, resembles a regenerating epithelium. The lack of different regions as in the previous biopsy and the uniformity in appearance of the present cell population suggest that the differentiated regions, which survived the









first heat treatment for a period of at least 4 days, were subsequently lost by desquamation. This is surprising since autoradiographic monitoring had shown that the differentiated cells in question continued to be engaged in macromolecular syntheses, and since there is ample evidence in the literature [21] that functional and morphological alterations induced in mammalian cells by supranormal temperatures in vitro are reversible when the cells are returned to

normal temperature. Nevertheless, the urothelium has now been completely reconstituted with a new population of small cells derived presumably from stem cells in the basal layer. The sporadic occurrence of mitotic cells tends to confirm this view since mitoses are infrequent [2] in normal bladder urothelium.

In the absence of serial biopsies, it remains unknown whether the differentiated cells under discussion were lost between days 4 and 13 after the first treatment or whether the loss occurred during and as a consequence of the second treatment. However, we saw earlier that a turnover of cells was already in progress by the 4th day after the first treatment in the regions constituted by small undifferentiated cells. It is hence probable that this turnover and regeneration extended to the differentiated regions as well after the 4 th day and before the commencement of the second course of treatment. Additional support for this assumption comes from the reports [2, 10] which refer to the remarkable ability of the urothelium to regenerate in experimental animals subjected to a variety of physical and chemical trauma. According to these reports, re-epithelialization can be brought about within a period of 4 to 5 days after the trauma. If the urothelium of elderly subjects respond likewise, a regenerated epithelium can be expected to be present within the 13 day period that elapsed between the first and second treatments.

The ultrastructure of the urothelium obtained 2 days after the short and long treatments would then reflect essentially the effects of the multiple perfusions of the second course of treatment on undifferentiated cells of a newly regenerated epithelium. The presence of vacuoles and the low electron density of the cytoplasm are indications of cell swelling and oedema, while the infiltration of the epithelium by leukocytes suggests inflammatory changes in the tissue. The most affected organelle was mitochondria, most of which were obviously disorganised and/or degenerating. Inhibition of mitochondrial activity and the resultant impairment of energy supply can be expected to have serious effects on cellular metabolism. These injuries are interpreted as demonstrating the damaging effect of the fractionated treatment on a newly regenerated urothelium. The extent of the damage is further indicated by the lack of incorporation of radioactive precursors. It is difficult, for obvious ethical reasons, to collect biopsies in order to be able to answer questions relating to the fate of this damaged epithelium. However, in view of what has emerged from this study, and the well known regenerative capacity of mammalian urothelium, one may expect the heat-damaged cells to be shed, and a fresh urothelium reconstituted in due course.

A course of fractionated treatment, by itself, induces general desquamation and leads to the loss of the normal pattern of progressive cytodifferentiation in the urothelium. This is abundantly clear in biopsies obtained from bladders only 3 days after being subjected solely to a course of multiple perfusions. Data do not exist, however, to determine the exact period during the treatment when the above events occurred. Extrapolating from an earlier observation that following a continuous 2 h treatment, general desquamation extending to all regions of the urothelium did not occur until at least 4 days after the treatment, one might assume that with multiple perfusions, a similar event is unlikely to have occurred until after several one-hour sessions. If this is the case, it would seem that regeneration also commenced *pari passu,* since a four to six layer thick urothelium with small undiffentiated cells came to be formed within a short period of only 3 days after the course of multipe perfusions ended.

With the above-mentioned thick urothelium continuing to lose cells by desquamation, it is surprising to find a few cells displaying rather extensive endoplasmic reticulum; the possible significance of this will be discussed later. Nevertheless, there is evidence of damage to mitochondria such as increased electron opacity of the matrix and reduced cristae. A small proportion of mitochondria with more pronounced alterations are presumably damaged beyond recovery. Autoradiographic evidence reveal continuing RNA and protein syntheses which lead to the conclusion that impairment of mitochondrial function as a whole was not so drastic as to cause an arrest of metabolic activities in the cells, although possibly these activities may have suffered a degree of inhibition.

Examination of urothelium at an interval of 10 weeks after a course of fractionated treatment reveals several aspects of interest. Firstly, the tissue exhibits a kind of stable (desquamation having practically ceased), compensatory hyperplasia with as many as nine layers of cells. Secondly, the constituent cells show a lower nuclear-cytoplasmic

◀ **Plate VI.** Micrographs of regenerating urothelium 10 weeks after fractionated treatment

Fig. 20. Light micrograph showing about nine layers of cells in the hyperplastic urothelium (x 560)

Fig. 21. High power electron micrograph to show apparently normal mitochondria *(M)* and many vesicles *(V)* in the cytoplasm adjoining the luminal membrane. Some profiles of endoplasmic reticulum are also seen (x 25,500)

Fig. 22. Note the well developed state of the Golgi apparatus *(GA)* with cisternal as well as vesicular components. The Golgi complexes are situated in the antero-lateral regions of the cytoplasm (x 25,500)

Fig. 23. A cell with extensive endoplasmic reticulum *(ER)*. Normal mitochondria are present. *JC,* Junctional complex consisting of a tight junction and further below a desmosome (x 17,000)

ratio than that found in cells of the urothelium 3 days after the fractionated treatment. The cells also have very irregular nuclei and these features make it likely that this population of cells is different from that described before at the earlier time interval after the fractionated treatment. This would imply that the previous cell population with damaged mitochondria were lost by continuing desquamation after day 3 of the end of treatment and replaced in the subsequent weeks by cells proliferating from the basal layer.

The mitochondria in the hyperplastic urothelium appear normal and the cells also show signs of cytodifferentiation. There are Golgi apparatuses with multiple components and well developed endoplasmic reticulum. The latter, however, must be regarded as an unusual feature because normal urothelial cells possess [12] little of this membranous system. A well developed ER system is generally looked upon as an expression of cellular differentiation but it remains possible that, both in this and the previous biopsy, its prominence indicates synthesis of some secretory proteins induced by heat-activated genes. It has been shown recently [22 and references cited therein], that several systems including cultured mammalian cells respond to heat shocks by synthesising a preferential set of proteins. The follow-up biopsies obtained at 10 and 12 week intervals after the fractionated treatment indicate that this length of time elapses before desquamation subsides and the regenerated urothelium attains some stability. Only further examination of post-treatment biopsies at later intervals will provide information relating to the mode of regression of hyperplasia in the regenerated urothelium and the restoration to it of ultrastructural normality with large, well-differentiated superficial cells. In a recent report, Likourinas et al. [15] have reported that, following gross destruction of urothelium by intravesical infusion of formalin, it took at least 6 months for the reappearance of a normal urothelium.

There are many reports in the literature dealing with the effects of higher than optimal temperatures on organisms, cells and tissue systems treated under a variety of conditions and end points. In these investigations [for references, see 1, 7 and 9] many targets have been identified; they include inhibition of synthesis of nucleic acids and proteins, alterations of cellular membranes leading to increased permeability and consequent ionic disturbances, damage to mitochondria leading to inhibition of oxygen consumption and uncoupling of oxidative phosphorylation, alteration of cellular pH, and disruption of lysosomes. By comparison with the data available in the past reports, the temperature of 44 °C used in the present study and the periods of treatment employed seem to be within the range at which normal cells can be expected to recover from heat effects on return to normal temperature. The present results, however, show that even a short treatment leads to a gradual shedding of the urothelial cells, even though there were no indications that the heat shock caused any large scale pycnosis or thermal death of cells. The desquamation that invariably seems to have followed the heat treatments described in this report may then have to be viewed as a characteristic in vivo response of the epithelium to heat trauma. What is more significant, however, is the finding that the cell loss was in turn followed by regeneration. This implies the continued survival and unimpaired proliferative capacity of stem cells in the basal layer of the epithelium. It may be that because of their dormant state, these cells are less sensitive to hyperthermia than their differentiated progeny. This view finds support in an earlier observation by Palzer and Heidelberger [20] that HeLa cells in which DNA and protein syntheses were inhibited by chemicals were less sensitive to hyperthermia than control cells.

It is hoped that the present favourable findings on the in vivo response of normal urothelium to heat will encourage the continuation of efforts to use hyperthermia for treating cancers such as those of the urinary bladder.

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Dr. J. Jacob
Department of Genetics
West Mains Road
Edinburgh EH9 3JN
U.K.