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Cell replacement and differentiation in transitional epithelium: a histological and autoradiographic study of the guinea-pig bladder and ureter

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INTRODUCTION

Transitional epithelium occupies a position between the pseudostratified and truly stratified epithelia, and there has been much conjecture regarding the arrangement of the constituent cells, their behaviour during stretching of the epithelium, and the manner in which they undergo replacement and differentiation.

In a previous study on the guinea-pig (Martin, 1962) it was concluded that there are essentially three layers of cells in the epithelium, namely, a layer of small basal cells, a middle or intermediate layer of pyriform cells, which vary in size and height but retain connexion with the basement membrane by long thin cytoplasmic processes, and a layer of surface cells which are of large size and often bi- or multinucleate. During distension, the stretched epithelium of both bladder and ureter retains the essentially three-layer state; the surface cells become flattened and thus increase their surface area, whilst the underlying cells are not only flattened but are displaced sideways and may lie almost parallel to the basement membrane (Martin, 1962; Petry & Amon, 1966).

In contrast with stratified squamous epithelia, in which there is a rapid rate of cell replacement from the basal layer, study of cell kinetics in transitional epithelium has proved difficult. Mitotic figures are seldom seen in the adult bladder and very few cells are labelled in autoradiographs following the administration of [^{3}H]thymidine (^{3}H-T). Leblond & Walker (1956) summarized the conflicting views regarding cell replacement in the epithelium, namely, whether replacement is only from the deeper cells or whether the surface cells also divide, either by mitosis or amitosis, and from study of the rat they confirmed reports of previous workers that mitoses may be found in the surface as well as in the deeper cell layers. Although the pattern of mitotic activity in fetal and newborn tissues does not necessarily persist, study of the mouse bladder at these stages has shown that the mitotic activity is higher than in the adult and occurs in all cell layers (Walker, 1958).

In autoradiographs of the adult female mouse bladder following ^{3}H-T administration, Walker (1960) found only nine cells labelled in a population of 41 000, and for the same species Levi, Cowan & Cooper (1969) found a labelling index of about 1 %. In the rat, Messier & Leblond (1960) found a labelling index of about 0·5 % and noted wide individual variations and much irregularity of distribution of the labelled cells, which included some surface as well as deeper cells.

Although there is so little mitotic activity in the adult bladder, it has been shown in the adult mouse (Walker, 1959) and cat (McMinn, 1969) that marked proliferative activity follows within 24 hours after artificial ulceration, and Walker found that when ^{3}H-T was administered 18 hours after operation many cells were labelled some distance away from the ulcer margin, and some surface cells were labelled as well as the numerous deeper cells. He also observed large mitotic figures in some surface cells. Following administration of carcinogenic drugs to mice, Levi *et al.* (1969) found that mitotic activity occurred 24 hours later in the cells of all layers and among all ploidy classes in the bladder, and although the majority of cells labelled with ^{3}H-T were in the basal layer, some intermediate and surface cells were also labelled. These experimental results for the mouse, taken together with the observations on control rats and mice, indicate that in these species the cells of all layers in transitional epithelium are self-perpetuating.

In the previous study (Martin, 1962) it was shown that, if the guinea-pig bladder is acutely distended with normal saline for 5 minutes, mitotic figures appear in the epithelium 24 hours later, and are confined to the basal layer. It seemed likely, therefore, that if ^{3}H-T were administered 24 or more hours after acute distension, a considerable number of cells would be labelled and their site would confirm the progenitor zone. Furthermore, if animals were killed at intervals following the ^{3}H-T injection, the fate of the labelled cells could be followed and thus provide information on the mode of cell replacement and differentiation.

MATERIALS AND METHODS

Eight control guinea-pigs, two aged 4 weeks and six aged 8 weeks, were given a single intraperitoneal injection of ^{3}H-T (1uC/g body weight) and killed 50 minutes later. The bladder and middle portions of the ureters were fixed in 80% alcohol, embedded in paraffin and sectioned at 5 μm. Some sections were stained with H. & E. for general histological study and others were layered for autoradiography, using Eastman-Kodak film strip. After 4–6 weeks, depending upon the results with trial sections, they were developed and counterstained with either toluidine blue or a modification of Harris's haematoxylin.

For the experimental series, 45 female guinea-pigs of about 8 weeks of age were employed. Acute distension of the urinary bladder was effected under direct vision, as follows. Under ether anaesthesia, the abdomen was opened by a mid-line incision and a glass catheter was then introduced into the bladder through the urethra. The catheter was made by drawing out a piece of glass tubing and curving the drawn end, which was tapered and about 2 cm long. After emptying the bladder, the catheter was withdrawn and attached by a short length of rubber tubing to a 10 ml syringe charged with sterile normal saline. After filling the catheter with saline to expel the contained air, it was re-introduced into the bladder and the latter filled with saline until the intravesical pressure caused the syringe plunger to move back a little when the thumb retaining it was removed. About 5–6 ml of saline was normally required to obtain this degree of acute distension, and the distension was maintained for 5 minutes in all cases, after which the bladder was emptied and the abdomen closed.

Since the previous study (Martin, 1962) had shown that mitotic figures appear in

the epithelium 24 hours after operation and are also present after 48 hours, some of the animals were given an intraperitoneal injection of ^{3}H-T at the 24 hour and others at the 48 hour period after operation. A very high labelling index was found compared with the controls, so a series of 10 animals was studied to determine for what length of time after operation the mitotic and DNA synthetic activity continued. These animals were killed in small groups from the fourth day after operation, and each received an injection of ^{3}H-T 50 minutes before death.

To study the fate of the labelled cells, 35 animals were given ^{3}H-T either 24 or 48 hours after operation and killed at intervals. This part of the investigation was carried out in two stages. The first was a short-term study in which 25 animals were killed in groups at times ranging from 50 minutes to 9 days after the ^{3}H-T injection. Following the observations made, a long-term study was undertaken on 10 animals. In this group the ^{3}H-T was always given 24 hours after operation, and the animals were killed at intervals of 4, 6, 8, 11, 12 and 16 weeks after the injection. In addition, two control female guinea-pigs were given ^{3}H-T and killed after 7 and 12 weeks respectively.

During study of the short-term series, some of the animals were catheterized daily up to the 8th post-operative day. Following centrifugation of the urine, smears were made from the deposit, and these were fixed in 80% alcohol for autoradiographic study of possible cellular content.

RESULTS

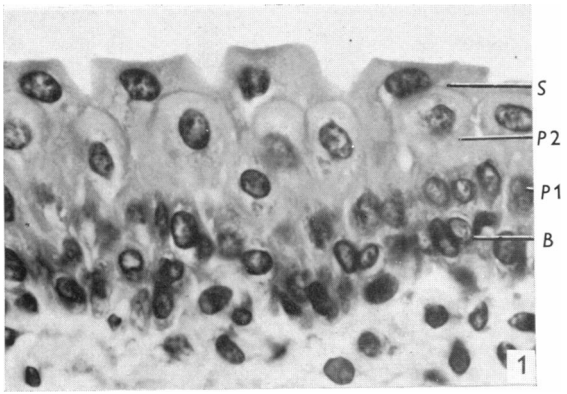
The control bladder and ureter

Structural features

Individual cell outlines are not clearly defined in routine histological preparations of transitional epithelium, but as already stated, there is evidence that the epithelium is essentially three-layered. Before the autoradiographic findings can be interpreted it is necessary to consider further the cellular interrelationships.

As shown in Fig. 1, the basal layer (*B*) consists of a row of small cells, and it will be shown that these are the progenitor cells in the adult guinea-pig. The pyriform cells compose the intermediate layer and, although they vary in size and height, each is connected by a thin cytoplasmic process to the basement membrane. The deeper, smaller cells usually have pointed apices (*P*1 in Fig. 1), whilst the largest cells have rounded apices that fit into indentations of the surface cells (*P*2 in Fig. 1). It is due to the varying lengths and sizes of the pyriform cells, with consequent differences in position of their nuclei, that the impression is gained from routine preparations that there are several strata of pyriform cells. In the rat and mouse, however, the pyriform cells do not vary much in size and consequently the epithelium is lower in depth and more obviously three-layered.

The surface cells (*S* in Fig. 1) form a distinct layer. They vary in size and some of them reach enormous proportions; Dogiel (1890) considered them to be amongst the largest vertebrate cells. They are not infrequently binucleate and the very large ones are multinucleate (Figs. 2, 3). The indentations on their deep surfaces (preserved when the cells are isolated) have given them the apt name of 'umbrella cells'. From their EM study of sections cut tangentially, Petry & Amon (1966) found evidence that these cells, like the pyriform cells, are connected to the basement membrane by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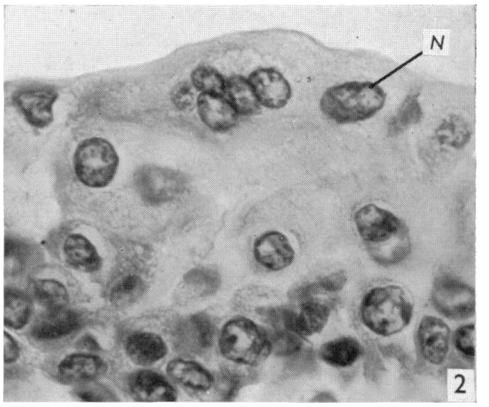
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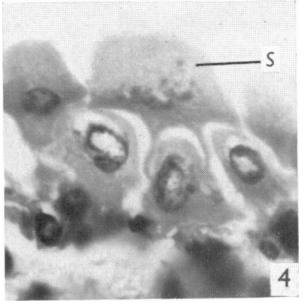
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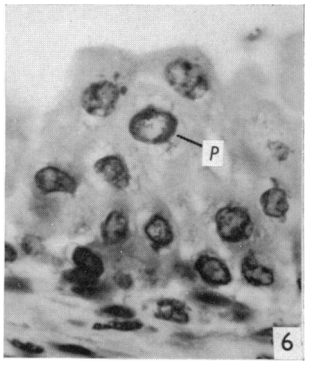
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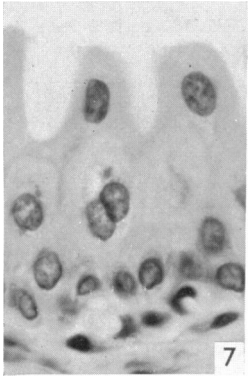
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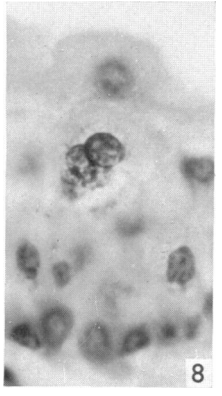
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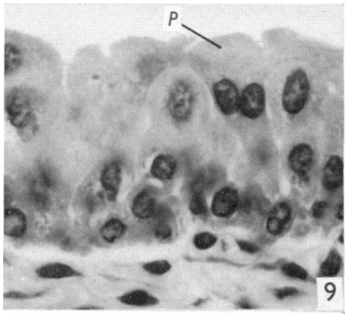
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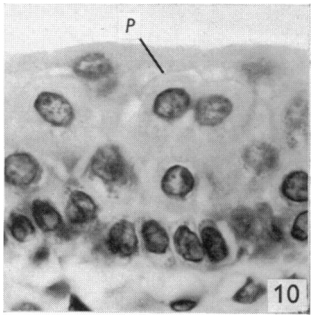
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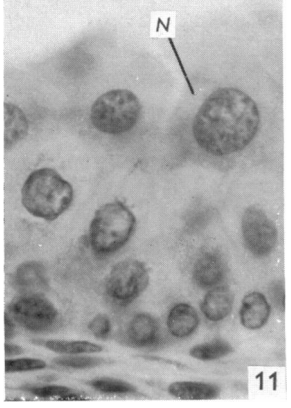
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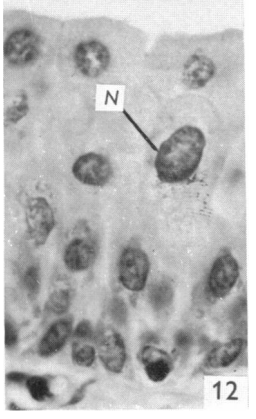
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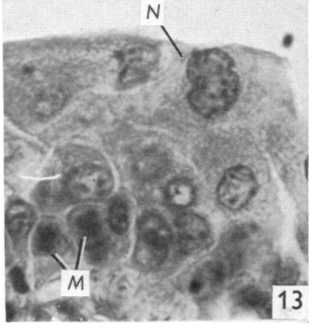
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thin cytoplasmic processes, and since the larger cells have several processes they suggested that they are formed by fusion of pyriform cells. Although such processes may occasionally be sectioned near the cell body they are too thin and tortuous to be traced very far between the pyriform cells in routine sections, but if the cells become separated, they may be seen occasionally (Fig. 4). Having established that all cells of the epithelium are attached by cytoplasmic processes to the basement membrane, Petry & Amon proposed that the epithelium should be considered as multilayered rather than stratified, and they pointed out that the fine processes are probably important in relocating the cells after their displacement during stretching.

In the present study, examination of a large number of H. & E. sections revealed that some of the large pyriform cells are binucleate or even multinucleate (Figs. 7, 8) and, furthermore, morphological appearances can be found suggesting transformation of pyriform cells to surface cells. In some instances a pyriform cell is seen extending into the surface layer and it may be already binucleate (*P* in Fig. 9). In other instances it appears that pyriform cells have gained the surface layer and are uniting over another pyriform cell, and if one or more of them is already binucleate, a multinucleate cell would result (Figs. 5, 6). Evidence of an early stage of fusion between adjacent pyriform cells with partial breakdown of their apposing cell walls may be encountered, though rarely, in routine preparations (*P* in Fig. 10). These

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Figs. 1–14. Haematoxylin and eosin preparations of guinea-pig bladder.

Fig. 1. The structure of transitional epithelium. Note the small basal cells (*B*), the lower and upper pyriform cells (*P*1, *P*2) and the surface cells (*S*). The cells increase markedly in size towards the surface and so do their nuclei. Each surface cell fits over several pyriform cells. × 600.

Fig. 2. In this somewhat tangential section is seen an enormous multinucleate surface cell containing a cluster of five closely set nuclei. The much larger nucleus (*N*) lying apart from this cluster belongs to the same cell. × 600.

Fig. 3. A large detached surface cell containing six nuclei. The indentations indicate regions of moulding to adjacent cells. × 600.

Fig. 4. Bladder, half emptied after acute distension. Cell separation reveals that the large multinucleate surface cell (*S*) is not only moulded over the underlying pyriform cells but also possesses fine cytoplasmic 'tails' which pass between them. × 600.

Fig. 5. Two upper pyriform cells, one already binucleate, are arching over two other pyriform cells and uniting in the surface layer. × 600.

Fig. 6. Two upper pyriform cells are arching over a very large, adjacent pyriform cell (*P*) and uniting in the surface layer. × 600.

Figs. 7, 8. In Fig. 7 is seen a binucleate upper pyriform cell and in Fig. 8 a large multinucleate pyriform cell. × 600.

Fig. 9. A large binucleate pyriform cell (*P*) is entering the surface layer. × 600.

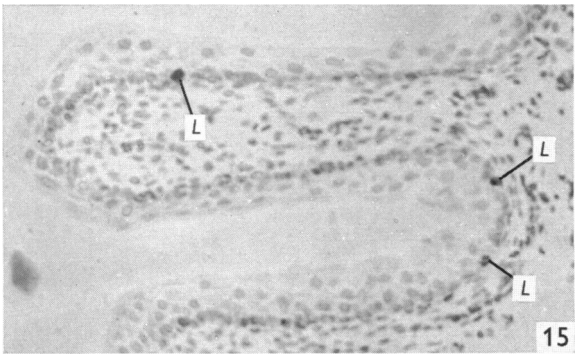
Fig. 10. Two pyriform cells (*P*) appear to be uniting and their nuclei lie close together. × 600.

Fig. 11. The nuclei of the upper pyriform and surface cells are often very large (compare with basal nuclei); the surface cell nucleus (*N*) is gigantic. × 600.

Fig. 12. A very large nucleus (*N*) is seen in a huge upper pyriform cell. × 600.

Fig. 13. The very large nucleus of a surface cell (*N*) shows an equatorial constriction, possibly indicating nuclear fusion. The bladder was acutely distended 24 hours previously and shows mitoses (*M*) in the basal layer. × 600.

Fig. 14. The nuclei of a binucleate surface cell (*N*) appear to be undergoing fusion, resulting in an 'hour glass' appearance. × 1000.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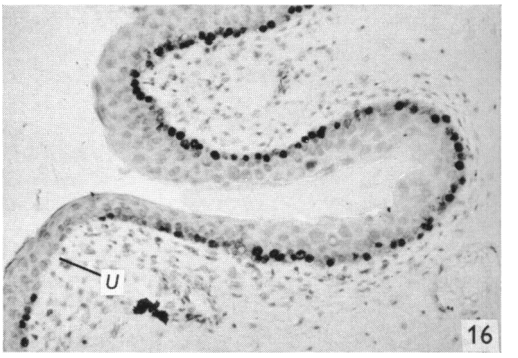
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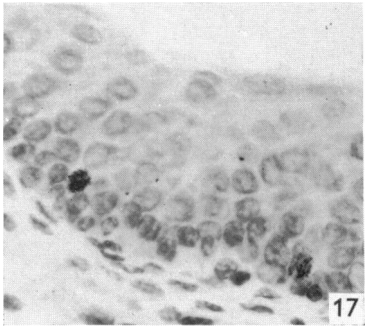
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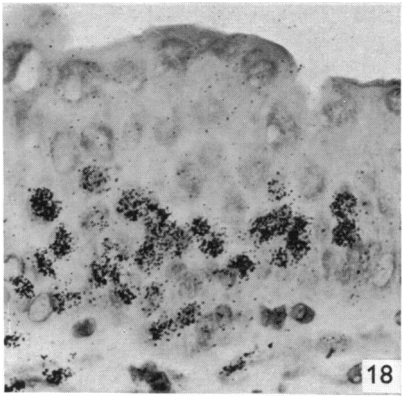
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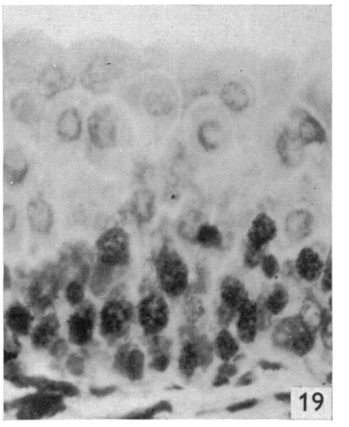
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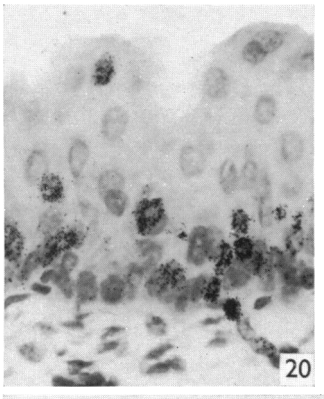
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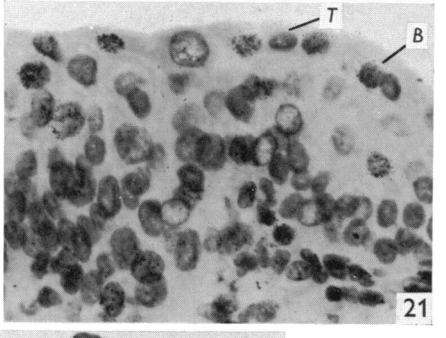
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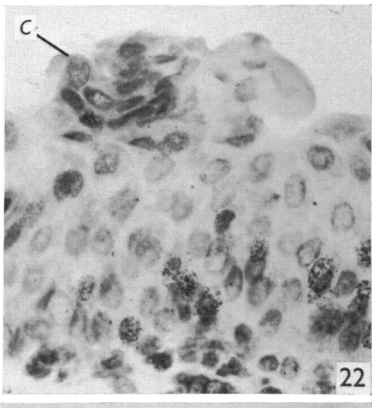
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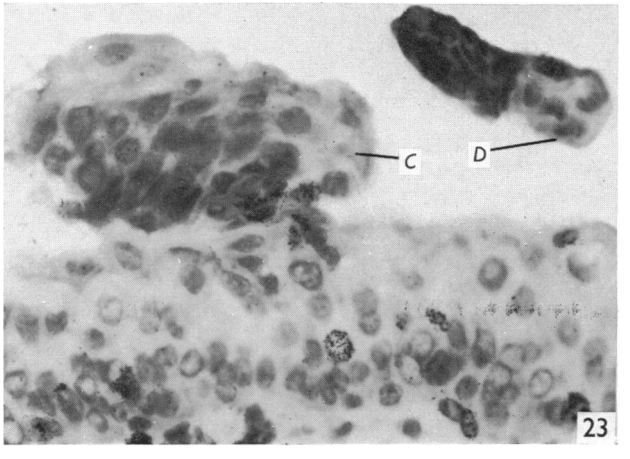
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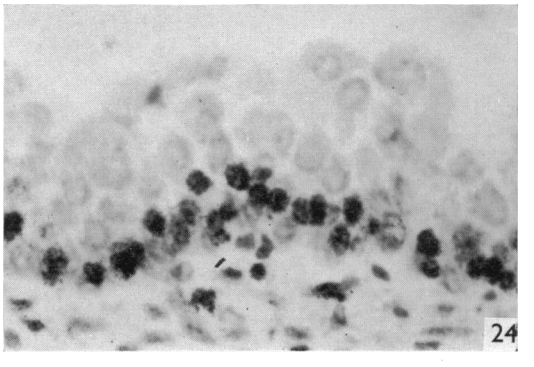
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morphological observations, although suggestive that cell fusion occurs, need support from autoradiographic studies, and these will be presented later.

A further notable feature of the epithelium is the presence of very large nuclei in some of the surface and large pyriform cells (*N* in Figs. 11, 12). As a result of the autoradiographic studies, attention was directed to these nuclei, since evidence became available that they are probably formed by nuclear fusion. Not uncommonly, some of these nuclei show marked indentations and occasionally a well-marked central constriction is seen, an appearance which in the past has often been regarded as evidence of amitosis but could well be the result of recent nuclear fusion (*N* in Fig. 13). The nuclei of binucleate cells, even of very large ones, commonly lie very close together and in rare instances the nuclear membranes appear to be fusing, which results in a 'dumb-bell' appearance (*N* in Fig. 14).

In the guinea-pig ureter, there are more rows of nuclei in the lining epithelium than in the bladder and this is due to greater variation in the height of the pyriform cells since, as noted earlier, only three layers of cells are seen in the distended ureter. In this species, the pyriform and surface cells do not reach large proportions and are seldom binucleate.

Mitotic and labelling indices

Mitotic figures were very scarce in the control material, and were confined to the basal layer. In autoradiographs, the scanty labelled cells were also confined to the

Figs. 15–24. Guinea-pig autoradiographs following [^{3}H]thymidine (^{3}H-T).

Fig. 15. Control bladder, 4 days after ^{3}H-T. Very few cells are labelled (*L*) and they are in the basal layer. × 150.

Fig. 16. Bladder, distended 24 hours prior to ^{3}H-T administration. Regions where many basal cells are labelled are separated by regions where few are labelled (*U*). × 95.

Fig. 17. Mid-ureter, 3 days after ^{3}H-T and 4 days after bladder distension. Only a few basal cells are labelled, whereas the bladder of this animal showed many labelled cells both within and above the basal layer (Fig. 19). × 375.

Fig. 18. Bladder, 2 days after ^{3}H-T and 3 days after distension. The epithelium shows increased cellularity and contains many labelled cells, which are no longer confined to the basal layer. Many connective tissue cells are labelled. × 375.

Fig. 19. Bladder, 3 days after ^{3}H-T and 4 days after distension. The epithelium shows increased cellularity and many cells are labelled. Some labelled nuclei now lie near the middle of the epithelium. × 375.

Fig. 20. Bladder, 5 days after ^{3}H-T and 7 days after distension. The nucleus of a surface cell is now labelled. × 375.

Fig. 21. Bladder, 5 days after ^{3}H-T and 7 days after distension. In this field several surface cells are labelled. In the trinucleate cell (*T*) two nuclei are labelled and in the binucleate cell (*B*) one nucleus is labelled. The epithelium shows disorganization and increased cellularity. × 375.

Fig. 22. Bladder, 5 days after ^{3}H-T and 7 days after distension. The epithelium shows increased cellularity and is somewhat disorganised. A clump of cells (*C*), some of which are degenerate, is detaching from the epithelium. × 375.

Fig. 23. Bladder, 5 days after ^{3}H-T and 7 days after distension. A large clump of cells (*C*), which contains both degenerating and labelled cells, has almost detached from the epithelium. In the detached clump (*D*) many cells are degenerate. × 375.

Fig. 24. Bladder, 8 days after ^{3}H-T and 9 days after distension. The epithelium is now of normal appearance, and the labelled cells are confined to the basal and lower pyriform cell layers. × 375.

Table 1. *Mitotic and labelling indices; control guinea-pigs*

	Bladder		Ureter.
	Labelling per 1000 basal cells	Mitoses per 1000 basal cells	Labelling per 1000 basal cells
4 week old	24	2	5
	23	1	16
8 week old	28	0	11
	17	0	9
	14	0·2	19
	11	0·4	—
	7	0	8
	3	0	5
Average for 8 week old	13	0·1	10

basal layer (*L* in Fig. 15). The mitotic and labelling indices in Table 1 are expressed as the number of mitoses and labelled cells per 1000 basal cells. At least three sections were examined from each animal, so that each estimate was made from counts of approximately 12000 basal cells.

As shown in Table 1 the mitotic and labelling indices were a little higher in the younger (4 weeks) than in the older (8 weeks) animals, suggesting a slightly greater rate of cell replacement, but it is clear from the very low figures for all the controls that the replacement rate is very slow. The average values for the 8 week animals, namely, a mitotic index of 0·01 per 1000 basal cells and a labelling index of 13 per 1000 basal cells are to be compared with the figures for the experimental groups, which were approximately 8 weeks old.

Short-term studies

Following acute bladder distension, 16 guinea-pigs received a single injection of ^{3}H-T after 24 hours whilst nine received the injection after 48 hours. Of the former group, four were killed 50 minutes after the injection to determine the 'immediate' labelling index, whilst the remaining 21 animals were killed in groups throughout a period of 9 days following the injection. The mitotic and labelling indices are summarized in Table 2, where the average values for each group are given. In Table 3 are shown the results of the experiments undertaken to determine for what period after the operation the increased mitotic and DNA–synthetic activity continues.

The immediate labelling index determined at the 24 hour period after operation gave an average value of 206 per 1000 basal cells, which was approximately 16 times the average control value. The average mitotic index was 3·6 per 1000 basal cells, and although very high in comparison with the control value, was very low in relation to the labelling index.

It will be seen from Table 2 that the labelling index was much higher when ^{3}H-T was administered at the 24 hour period after operation than at the 48 hour period; in fact, some of the latter figures were not much above the control values. Table 3 shows that there is, in fact, a rapid decline in the effectiveness of the stimulus. By the

Table 2. *Average mitotic and labelling indices at intervals following bladder distension*

(^{3}H-T administered either at 24 hours or at 48 hours* after operation)

Time after ^{3}H-T injection	Bladder			Ureter	
	Labelling per 1000 basal cells	Total cells labelled per length of 1000 basal cells (% above basal layer)	Mitoses per 1000 basal cells	Labelling per 1000 basal cells	Total cells labelled per length of 1000 basal cells (% above basal layer)
50 minutes	206	206 (Nil)	3·6	16	(Nil)
2 days	237	395 (40%)	2·6	40	(Nil)
3 days	241	376 (36%)	0·2	57	65 (13%)
4 days	169	307 (44%)	0·3	94	128 (27%)
	14*	18 (23%)*		16*	—
5 days	433	708 (39%)	0·2	117	154 (24%)
	77*	155 (50%)*		30*	34 (7%)*
6 days	22*	33 (33%)*	0·0	41*	45 (11%)*
8 days	316	560 (44%)	1·0	85	119 (30%)
9 days	222	296 (25%)	0·0	12	(Nil)
	43*	82 (48%)*		21*	24 (12%)*
Average after 1st day, excluding*	270	407 (33%)		68	86 (21%)

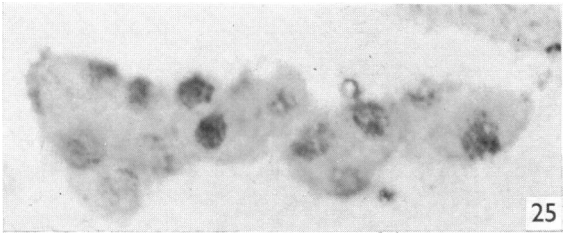
Table 3. *Mitotic and labelling indices*

(^{3}H-T administered at intervals following bladder distension)

Time elapsed post-op. before ^{3}H-T given	Bladder		Ureter.
	Labelling per 1000 basal cells	Mitoses per 1000 basal cells	Labelling per 1000 basal cells
4 days	46	2·4	4
5 days	23	0·4	3
6 days	4	0	2
7 days	3	0	5
11 days	1	0	4
11 days	1	0	5
21 days	6	0	3
21 days	4	0	21
1 year	3	0	4
1 year	20	0	9

4th to 5th day after operation the mitotic and labelling indices returned to control levels, and beyond that time the indices were in some cases even lower. It is thus clear that to obtain a maximal number of labelled cells, ^{3}H-T should be given 24 hours after operation.

From the labelling indices in Table 2, which were obtained when ^{3}H-T was given 24 hours after operation, it will be seen that the number of labelled cells was almost doubled 2 days later and that 40% of them had left the basal layer. From then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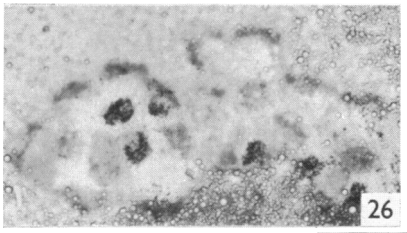
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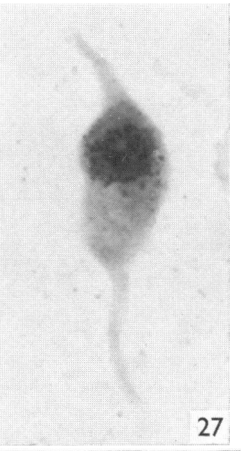
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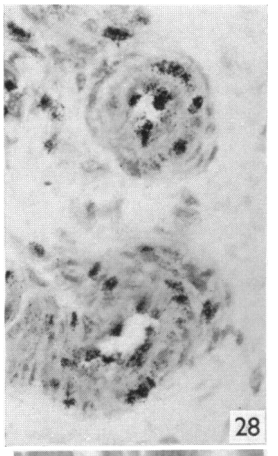
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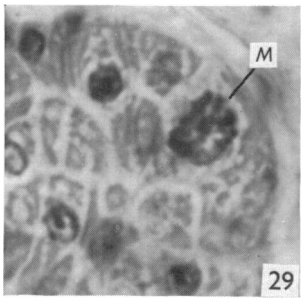

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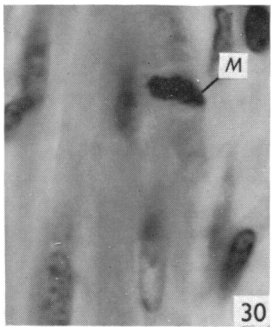

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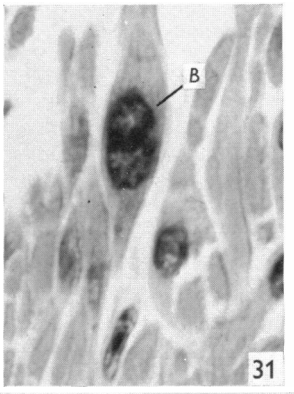

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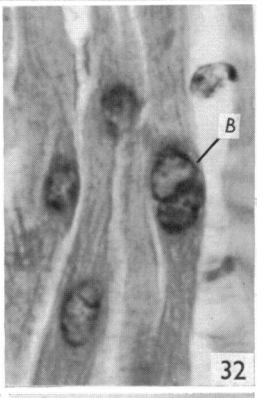

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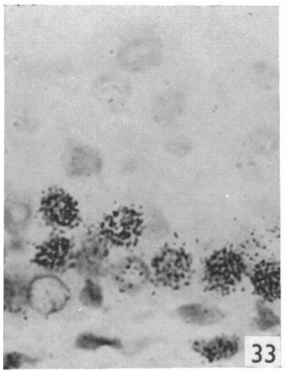
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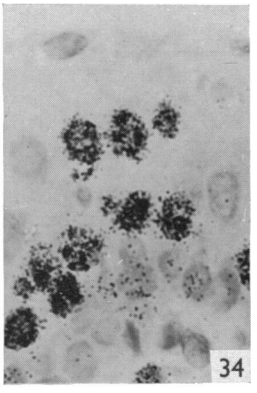
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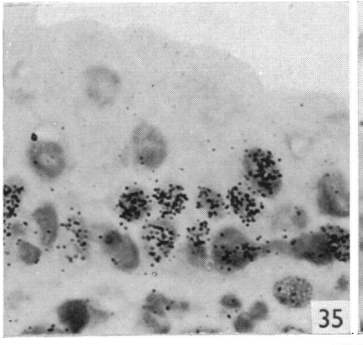
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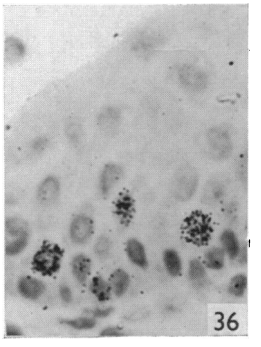
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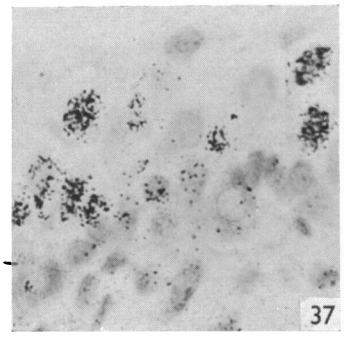
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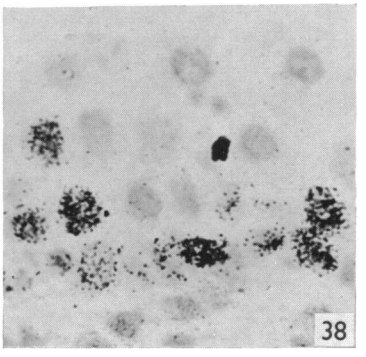
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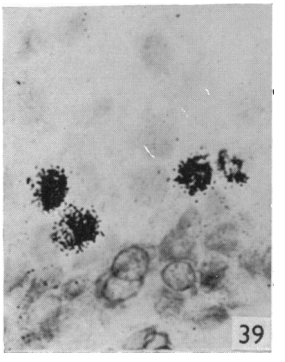
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onwards throughout the 9-day period there was no substantial change, apart from a temporary increase at the 5th day.

The ureter also showed a higher labelling index in the experimental group than in the controls, but it fell very far short of the bladder index. Although it is recorded in Table 2 that some of the labelled cells were above the basal layer, they were only marginally so. When sections of the intramural part of the ureter were encountered in the bladder wall, they showed a labelling index as high as that of the bladder.

As in control animals, the cells which immediately labelled 24 hours after operation were confined to the basal layer and, curiously, their distribution was always patchy; regions where most cells were labelled were separated by regions where few were labelled (see Fig. 16). Whereas in control animals the labelled cells were still confined to the basal layer after 4 days (Fig. 15), in the experimental groups 'migration' occurred by the second day after injection. Although at all periods the labelled cells were largely confined to the deeper half of the epithelium (Figs. 18, 19), in some areas a few labelled cells rapidly differentiated into upper pyriform cells and by the fifth day even a few surface cells were labelled (Figs. 20, 21).

After careful search, a very small number of labelled binucleate surface cells was found. Although both nuclei were labelled in some, in others only one of the pair was labelled (Fig. 21), which strongly suggests that such cells were formed by fusion of a labelled with an unlabelled cell. Further observations on this point will be made when dealing with the long-term studies.

Figs. 25–39. All illustrations are of guinea-pig material.

Fig. 25. Clump of cells from centrifuged urine smear, 2 days after ^{3}H-T and 3 days after bladder distension. Some nuclei are labelled. × 375.

Fig. 26. Clump of cells from centrifuged urine smear, 4 days after ^{3}H-T and 5 days after bladder distension. The cells are degenerating but some nuclei are well labelled. × 375.

Fig. 27. Pyriform cell with labelled nucleus, from centrifuged urine smear 3 days after ^{3}H-T and 4 days after bladder distension. Note the long cytoplasmic 'tail' of the cell. × 1000.

Fig. 28. Bladder, 5 days after ^{3}H-T and 6 days after distension. Many connective tissue cells are labelled, and also endothelial and smooth muscle cells of blood vessels. × 300.

Fig. 29. Bladder, 3 days after distension. Smooth muscle in cross-section showing a muscle cell in early metaphase (*M*). H. & E. × 1000.

Fig. 30. Bladder, 3 days after distension. Smooth muscle in L.S. showing a muscle cell in metaphase (*M*). H. & E. × 1000.

Figs. 31, 32. Bladder, 3 days after distension, showing large binucleate smooth muscle cells (*B*). H. &. E. × 1000.

Figs. 33, 34. Bladder, 4 weeks after distension and ^{3}H-T. Most labelled cells are equally labelled and still confined to the basal and lower pyriform cell layers, as at 8 days (see Fig. 24; lower power), but a few upper pyriform cells are labelled (Fig. 34). × 600.

Fig. 35. Bladder 6 weeks after distension and ^{3}H-T. The distribution of labelled cells within the epithelium is similar to that at 4 weeks (Figs. 33, 34), but now many of the basal cells show reduction of label. × 600.

Fig. 36. Ureter, 6 weeks after ^{3}H-T. Most labelled nuclei now lie slightly above the level of the basal layer. × 600.

Figs. 37, 38. Bladder, 8 weeks after distension and ^{3}H-T. Although in some regions most labelled cells are now above the basal layer (Fig. 37), in other regions a considerable number still remain within the basal layer and not all show reduction of label. × 600.

Fig. 39. Ureter, from same animal as in Fig. 38. Labelled nuclei now lie above the level of the basal layer. × 600.

Following such a marked increase in cell population within a short time, it might be expected that the epithelium would show some increase in thickness or some evidence of cell shedding, and indeed both features were observed. From the third day after operation, areas were seen where the cell population was greater than in adjacent areas, resulting in localized increases in epithelial depth with structural disorganization (Figs. 18–21), and it was particularly in these areas that evidence of cell shedding was observed, especially on the fourth and fifth days after operation. The zones of shedding presented appearances similar to those observed by Leblond & Stevens (1948) in the small intestine, where the epithelial cells become effete on reaching the tips of the villi and are shed, often in small clumps, the constituent cells of which usually show signs of degeneration.

The cell clumps undergoing extrusion were often of considerable size, though still in continuity with the parent epithelium. Usually, some cells within the clumps were already undergoing degeneration, and showed general shrinkage and nuclear pyknosis. Some of the cells, whether degenerate or not, had labelled nuclei (Figs. 22, 23).

Further evidence of cell shedding was provided by the autoradiographs prepared from smears of centrifuged urine. In these, shed cells were observed from the second day after operation and thus a little earlier than obvious evidence of shedding was seen in the sections. The shed cell clumps were of similar appearance to those in the process of extrusion from the epithelium, and again some, whether obviously degenerating or not, had labelled nuclei (Figs. 25, 26). Single shed cells were also found, and occasionally a well-preserved pyriform cell was labelled (Fig. 27), indicating the rapidity with which cells can differentiate following the operation, since such cells must have been derived from basal cells, labelled a few days previously.

In the animals examined 8 and 9 days after ^{3}H-T injection (9 and 10 days after operation respectively), the epithelium had returned to a quiescent state and showed no areas of thickening or cell shedding. A large number of labelled cells was still present in the epithelium and most of them were well labelled. They were confined to the basal and lowermost pyriform cell layers (Fig. 24), so it appears that the small number which had developed rapidly into upper pyriform and surface cells were among those shed from the epithelium. By the eighth day after operation, scarcely any shed cells were found in the centrifuged urine.

Response of the muscular and connective tissues

The connective and smooth muscle tissues of the bladder wall also showed a very marked increase in the number of labelled cells following the operation. Counts were made of all cells labelled throughout the connective and smooth muscle tissues in six control guinea-pigs, for comparison with the counts obtained for the experimental series. The results are shown in Table 4 and each count is the average from two sections.

In the control series, an extremely small number of cells was labelled within the vast population of connective tissue and smooth muscle cells; in the specimens studied, thirty was the maximum number. In the experimental series, the numbers varied from hundreds to well over a thousand per section and, although fairly generally distributed, they were somewhat more concentrated in the subepithelial

Table 4. *Total cells labelled in bladder wall, excluding epithelium*

Controls (50 minutes after ^{3}H-T)							
3, 4, 4, 8, 14, 30.							
Periods after ^{3}H-T (given 24 hours or 48 hours* after bladder distension)							
50 minutes	2 days	3 days	4 days	5 days	6 days	8 days	9 days
493	917	716	140	233*	197*	175*	25*
567	1805	1537	1509	281*			1217
1925				359			
				797*			
				1305*			

Periods after bladder distension before ^{3}H-T given						
4 days	5 days	6 days	7 days	11 days	21 days	1 year
727	136	375	257	52	22	27
				33	28	54

connective tissue than elsewhere. The labelled smooth muscle cells included those in the walls of arteries, and here the endothelial cells were also labelled (Fig. 28). Table 4 shows that the enhanced activity lasted for at least 7 days, compared with 4–5 days in the epithelium. A few mitotic figures were also seen in smooth muscle (Figs. 29, 30) and occasionally a large binucleate muscle cell was observed (Figs. 31, 32). Although mitotic figures appeared in the epithelium 24 hours after distension, they were first observed 48 hours after operation in smooth muscle.

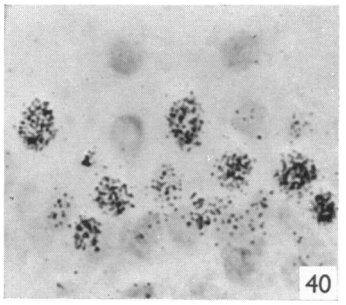
Long-term studies

Since it was shown that the epithelium returns to apparent normality by the 10th postoperative day, but still contains a large number of well-labelled basal and lower pyriform cells, animals were allowed to survive for much longer postoperative periods in order to trace the later history of these cells.

Following the observations made in the short-term study, all these animals were given a single intraperitoneal injection of ^{3}H-T 24 hours after acute bladder distension.

As shown in Table 5, the mitotic index remained at control level, and a very large number of labelled cells remained in the epithelium, throughout the long experimental period. In fact, the average number for all periods studied from 4 to 16 weeks was 379 per length of 1000 basal cells, which was only a little lower than the average of 407 for the 2 to 9 day labelling period. However, there was a steady rise in the number which 'migrated' from the basal layer until the 11 week period, after which the proportion above the basal layer remained constant at approximately 90%. In both the 7 and 12 week controls the labelled cells were very few in number and 80% had left the basal layer.

In the ureter, as shown in Table 5, the average number of cells labelled in the epithelium, calculated per length of 1000 basal cells, was 86 during the 2 to 9 day period and this was considerably higher than the average control value of 10. During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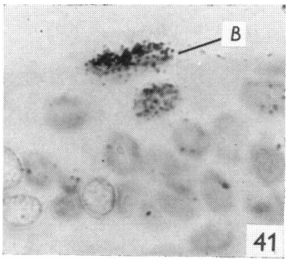

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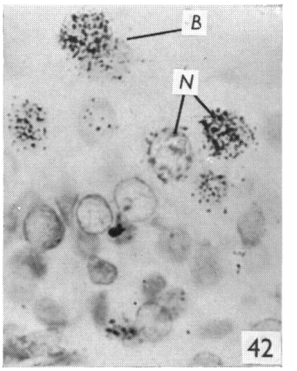

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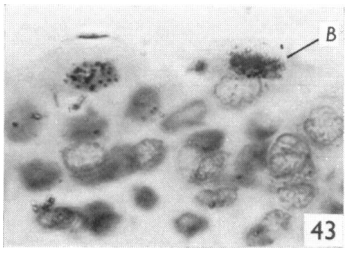

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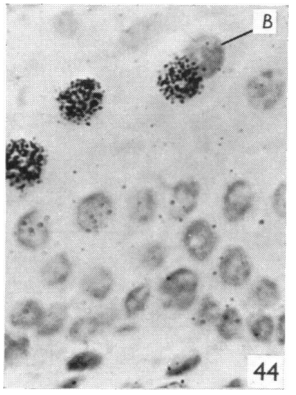

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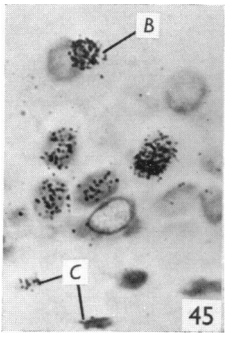

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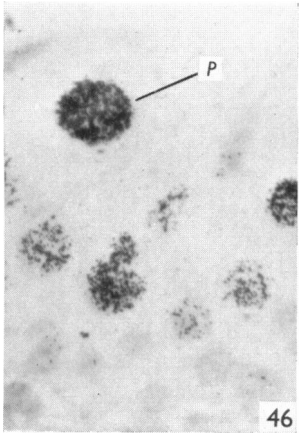

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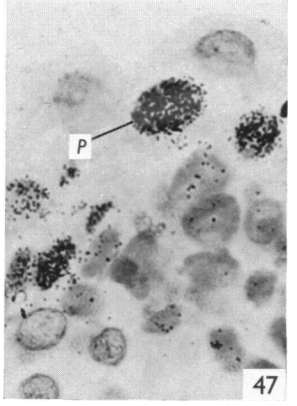

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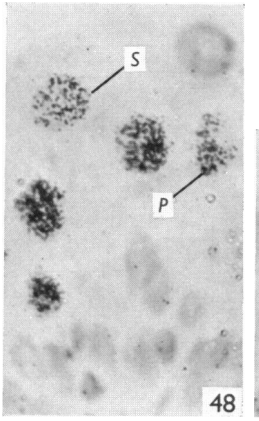

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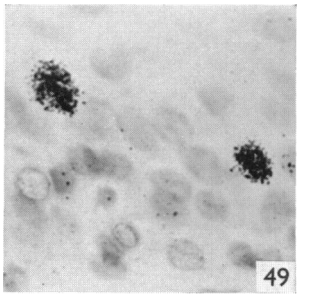
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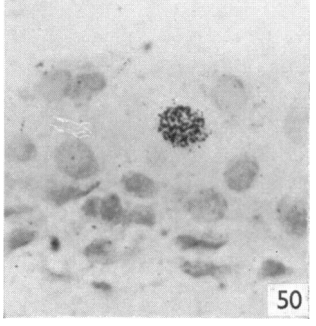
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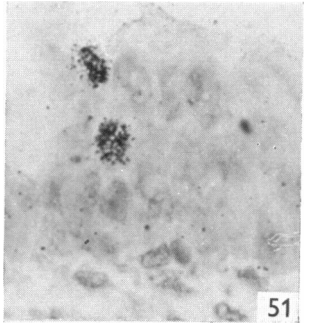
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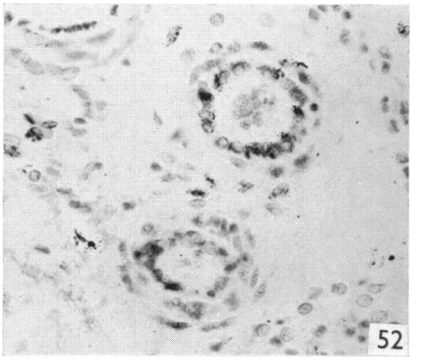
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the 4 to 16 week period the average number was 140, which suggests that during this later period some of the labelled cells had divided before leaving the basal layer. Most of the labelled cells had 'migrated' from the basal layer earlier than in the bladder and very few remained there after the 6th week. Although there were fewer labelled cells in the long-term controls, the results were similar.

The majority of the labelled cells in the bladder were still located in the basal and lower pyriform cell layers after 4 weeks (Fig. 33), but in places a few upper pyriform cells were labelled (Fig. 34). Furthermore, there was little variation in the degree of labelling.

After 6 weeks more upper pyriform cells were labelled than at 4 weeks, and many of the basal and some of the lower pyriform cells showed a lesser degree of labelling than those above them, indicating that division of labelled basal cells had now taken place (Fig. 35).

After 8 weeks it was apparent that a considerable proportion of the labelled cells had by this time 'migrated' from their original location in the basal and lower pyriform cell layers and had differentiated into large, upper pyriform cells. Those

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Figs. 40–52. All illustrations are of guinea-pig material.

Fig. 40. Bladder, 12 weeks after distension and ^{3}H-T. Many labelled cells remain in the epithelium and in places a few are still in the basal layer, but here the label is weak. × 600.

Fig. 41. Bladder, 12 weeks after distension and ^{3}H-T. The nuclei of a surface binucleate cell (*B*) are equally labelled. × 600.

Fig. 42. Bladder, 12 weeks after distension and ^{3}H-T. The nuclei of a surface binucleate cell (*B*) are unequally labelled. Note that adjacent cells deep to the surface layer show unequal labelling of their nuclei (*N*). × 600.

Fig. 43. Bladder, 12 weeks after distension and ^{3}H-T. Surface cells only are labelled in this field and the binucleate surface cell (*B*) shows labelling in only one nucleus. × 600.

Fig. 44. Bladder, 12 weeks after distension and ^{3}H-T. In this field only some large nuclei in the upper part of the epithelium are labelled and the surface binucleate cell (*B*) shows labelling in only one nucleus. × 600.

Fig. 45. Bladder, 12 weeks after distension and ^{3}H-T. Here some deeper cells are labelled and the surface binucleate cell (*B*) shows labelling in only one nucleus. Connective tissue cells (*C*) are still labelled. × 600.

Fig. 46. Bladder, 11 weeks after distension and ^{3}H-T. The gigantic upper pyriform cell (*P*) contains a very large nucleus which is overlaid by an enormous number of grains. × 600.

Fig. 47. Bladder, 12 weeks after distension and ^{3}H-T. The very large nucleus of an upper pyriform cell (*P*) is overlaid by an enormous number of grains. Compare with deeper labelled nuclei. × 600.

Fig. 48. Bladder, 12 weeks after distension and ^{3}H-T. Some of the large nuclei of these surface (*S*) and upper pyriform cells (*P*) are rather sparsely labelled. × 600.

Fig. 49. Ureter, 12 weeks after ^{3}H-T. Some well labelled cells are still present and all are now above the basal cell layer. × 600.

Fig. 50. Control bladder, 7 weeks after ^{3}H-T. The nucleus of an upper pyriform cell is well labelled. × 600.

Fig. 51. Control bladder, 12 weeks after ^{3}H-T. Some upper as well as lower pyriform cells are labelled. × 600.

Fig. 52. Bladder, 12 weeks after distension and ^{3}H-T. Many connective tissue cells are still well labelled and also endothelial and smooth muscle cells of blood vessels. Compare with Fig. 28 (5 days). × 250.

Table 5. *Mitotic and labelling indices after long intervals following bladder distension*
(^{3}H-T administered 24 hours after operation)

Time after ^{3}H-T injection	Bladder				Ureter		
	Basal cells labelled per 1000	Total cells labelled per length of 1000 basal cells	% above basal layer	Mitoses per 1000 basal cells	Basal cells labelled per 1000	Total cells labelled per length of 1000 basal cells	% above basal layer
4 weeks	184	461	60	1	43	71	40
	140	357	60	0	69	225	69
6 weeks	50	168	70	0	50	242	79
	117	457	74	0·2	15	94	84
	70	300	77	0	4	122	97
8 weeks	86	394	78	0·6	4	127	97
	69	330	79	0	3	166	98
11 weeks	82	820	90	0	4	122	97
12 weeks	17	191	91	0	9	146	94
16 weeks	35	315	88	0	3	83	96
Average total during 4–16 weeks		379				140	
Average total during 2–9 days		407				86	
			Long-term controls				
7 weeks	1	5	80	0	0	41	100
12 weeks	1	5	80	0	0	17	100

remaining among the basal and lower pyriform cells frequently, but not invariably, showed reduced labelling (Figs. 37, 38; compare with Fig. 33).

After 11 and 12 weeks, migration of the labelled cells was even more obvious, with a shift in position of a considerable number towards the free surface of the epithelium (Figs. 40–48). Most of those which remained in or near the basal layer now showed only about one quarter the number of grains overlying most pyriform cell nuclei of similar size, suggesting that they had divided twice (Fig. 40).

In addition to the considerable number of upper pyriform cells, some surface cells were also labelled at the 11 to 12 week periods. A search was therefore made for labelled binucleate cells, but it will be appreciated that their numbers were small, since normally only a small proportion of sectioned surface cells show more than one nucleus, and of these only a few could be expected to be labelled. Nevertheless, following examination of a large number of sections, labelled binucleate cells were encountered and the manner of their labelling is of considerable significance, since it provides evidence regarding their mode of formation.

A very few of these cells showed the nuclei equally labelled (*B* in Fig. 41), or unequally labelled (*B* in Fig. 42), but cells in which only one of the nuclei was labelled were seen much more frequently (*B* in Figs. 43–45). It is difficult to understand how this could result, other than by fusion of a labelled with an unlabelled cell.

It was noted earlier that some of the very large surface and upper pyriform cells may contain exceptionally large (polyploid) nuclei. In the autoradiographs, some of these nuclei were overlaid by a very large number of closely-set grains (*P* in Figs. 46, 47). Counts showed that such nuclei were associated with at least twice the number

of grains that were found in relation to maximally labelled nuclei of normal size; for example, the large nucleus in Fig. 46 gave a grain count of 294, whilst the average for 10 maximally labelled nuclei of normal size was 128 (range 114–144). The latter figure was near the average for well-labelled basal cells in the short-term studies when animals were sacrificed within an hour of ^{3}H-T administration. Even in these preparations the degree of labelling does vary, but the average grain count of 100 well-labelled basal cells was 140, and the maximum was 170.

These results suggest that nuclear fusion, in addition to cell fusion, is involved in the formation of the polyploid cells, since the only source of their label was from the small basal cells. Not all the large labelled nuclei were overlaid by a large number of grains. Some of them (e.g. *P* and *S* in Fig. 48) showed a sparse overlay which could be due to fusion of a labelled with an unlabelled nucleus.

In the long-term bladder controls, the labelling index was the same at 12 as at 7 weeks. Examination of 12 sections from each animal revealed that the distribution of the labelled cells was also much the same, but the small number of cells available for study made assessment of their relative distribution somewhat difficult. The majority of labelled nuclei lay near the middle of the epithelium but some were present in the upper pyriform cell layer (Figs. 50, 51). None was found in the surface cell layer, even after 12 weeks. Most nuclei in a basal or parabasal position showed a weaker label than those more superficially placed, suggesting that division of labelled basal cells had occurred prior to the 7th week. In the control ureter the labelled nuclei lay near the middle of the epithelium at both periods (Fig. 49).

Muscular and connective tissues

Throughout the long-term study, large numbers of labelled cells remained in these tissues, ranging from several hundreds to over 3000 and, as found in the short-term study, there was a wide variation between the individual animals at each time period. Throughout the period of study the majority of these cells, including the smooth muscle and endothelial cells of blood vessels, remained well labelled (Fig. 52).

DISCUSSION

It has been shown that 24 hours after acute distension of the guinea-pig urinary bladder, the basal layer of the lining epithelium shows increased mitotic activity and the DNA labelling index of that layer increases approximately 16-fold. This increased activity continues for 4–5 days.

Several investigators have reported a similar response in both this and other tissues following trauma. For example, following artificial ulceration of the bladder epithelium, mitotic activity occurred after 24 hours in the mouse (Walker, 1959) and cat (McMinn, 1969), and declined to control level by the fourth day in the mouse and by the end of the first week in the cat. Noltenhuis, Kempermann & Oehlert (1964) found that within 12 hours after unilateral nephrectomy the tubules of the remaining kidney showed a marked rise in mitotic activity and in DNA labelling. The labelling increment lasted for 6 days and at its peak reached 20 times the control level. Jacoby (1953) reported that 24 hours after the common bile duct was ligated in the guinea-pig, the lining epithelium of the gall bladder exhibited a burst of mitotic activity,

which returned to control level after the fourth day: this activity resulted in cell crowding and the formation of more than one layer of cells in places. He later reported (1959) that the connective tissue, smooth muscle (including the tunica media of arteries) and vascular endothelium were also involved in the response, which in these situations commenced a day later and lasted for several days longer than in the epithelium. The present study showed the same sequence in these tissues in the wall of the urinary bladder. Recently, McGeachie (1971) has recorded that intact smooth muscle cells adjacent to a crush injury to the muscle undergo mitosis by the third day. This is an interesting phenomenon since adult smooth muscle does not show mitotic activity; in fact, Post & Hoffman (1968) classify it as non-replicating.

It is difficult to say why a short period of overdistension of the bladder should call forth such a marked response in both the lining epithelium and the mesodermal tissues, but it is likely that stretching beyond physiological limits damages at least some of the cells and it has been proposed that damaged cells release 'evocators' which induce their neighbours to divide (Leblond & Walker, 1956). In fact, it has been shown in cross-circulation experiments between hepatectomized and normal rats that, after a lag period of about 19 hours, the liver of the normal as well as that of the operated rat shows increased DNA synthesis (Moolten & Bucher, 1967).

It is thus becoming clear that a variety of tissues show a similar pattern of response to trauma of various kinds; after a lag period of one day or a little less there is increased proliferative activity, which declines to control level within the succeeding week. The lining epithelium of the urinary bladder followed this pattern in the present study. Certain other features of the early stages of the response also need consideration before discussing the results of the long-term study.

The sudden increment in cell population was followed by localized increases in epithelial depth with structural disorganization, but this was soon corrected by cell shedding. It is not surprising that there were individual differences in the mitotic and labelling indices, and although studies of the cell cycle phases were not within the compass of the present investigation, this period of proliferation is of interest.

The average labelling index obtained 24 hours after operation was approximately sixty times the average mitotic index, which is a very high ratio compared with that of the proliferative cell compartment in tissues which undergo constant renewal. In most of the latter, DNA synthesis (S phase) occupies about 7–8 hours and mitosis (M phase) about 1 hour (Thrasher, 1966; Post & Hoffman, 1968), so that the ratio of labelling to mitotic indices is approximately seven in these tissues.

It is unlikely that the very high ratio found in transitional epithelium under the present circumstances would be accounted for by marked differences in S and M phases since the S phase has been estimated to last 5 hours in the normal ureter (Blenkinsopp, 1969) and 6 hours in the bladder following artificial stimulation of mitosis (Levi *et al.* 1969). It is possible, however, that it could result either from the brief and somewhat erratic nature of the proliferative response or from failure of a proportion of the cells synthesizing DNA to undergo division.

The latter possibility merits consideration since not all authors share the view of Messier & Leblond (1960) that, with few exceptions, cells which incorporate ^{3}H-T are about to divide. Pelc (1963) pointed out that in a number of cell populations there is a marked excess of DNA incorporation over cell division; for example, in the

mouse seminal vesicle he found the ratio of labelling to mitotic indices to be eighty and only 13 % of labelled cells had divided after 24 hours. In addition, a number of cells in tissues commonly classified as non-replicating (e.g. brain, smooth and cardiac muscle) show evidence of DNA synthesis at a low rate (Pelc, 1963; Post & Hoffman, 1968).

In the present study, the average number of labelled cells was approximately doubled by the second day after the ^{3}H-T pulse label and there was neither a significant change in their number nor obvious differences in their degree of labelling throughout the succeeding week. It would appear, therefore, that the labelled cells underwent a single division, and thus the high ratio of labelling to mitotic indices was probably determined in some way by the brief, erratic nature of the proliferative response.

The most significant finding relative to the long-term study was that within about 10 days following the pulse labelling the epithelium returned to structural normality, yet continued to contain a large number of well labelled cells, almost entirely confined to the basal and lower pyriform cell layers. Presumably the small numbers of labelled cells which rapidly differentiated into upper pyriform and surface cells were among those shed from the epithelium.

The long-term study showed that the labelled cells migrated very slowly from the deeper part of the epithelium and differentiated into upper pyriform and surface cells. The migration was irregular, so that at later periods labelled cells were scattered at all levels within the epithelium and even after 12 weeks, when most of them were located in the upper part of the epithelium, a few still remained in the basal layer. The small numbers which had by then reached the surface layer were probably derived from the initially labelled lower pyriform cells, and it is likely that the time taken for basal cells to migrate and differentiate into surface cells would be considerably longer. In the control bladders and ureters the few labelled cells had migrated no further than the middle of the epithelium after 12 weeks.

Although most of the migrating cells remained well labelled, suggesting that they had not undergone a second division, many of those which remained in the basal layer for 6 weeks showed a reduced amount of label compared with those above them, and the few which remained for 12 weeks showed only about a quarter of the label shown at the early periods. It seems probable, therefore, that the cycle time of the basal cells is in the region of 6 weeks (1000 hours). A much longer cycle time (8000 hours) was estimated by Blenkinsopp for the basal cells of the mouse ureter, but the replicating population available for study was very small.

There remains to be considered the mechanism by which the small, diploid basal cells transform during migration into the very large pyriform and surface cells, which contain large (polyploid) nuclei and often more than one nucleus. In the past, transitional epithelium has not proved a favourable tissue for this type of investigation, but in other populations containing binucleate and polyploid cells, particularly the liver cells and cells in tissue culture, the mechanism has been extensively studied. The possible processes involved have been summarized and discussed by Beams & King (1942), Wilson & Leduc (1948) and Carriere (1967), and they include endomitosis, cryptomitosis, amitosis, mitosis with failure of cytokinesis, and also fusion of cells and of nuclei.

There is considerable evidence that in many, if not all cases, the formation of a

binucleate cell is the first step in the change to higher ploidy. The sequence is suggested by the observation that in the neonatal liver, uninucleate diploid cells predominate and mitoses are present. As the animal matures, however, mitoses become rare, whilst binucleate and polyploid cells appear in increasing numbers, so that the adult population shows a variety of ploidy levels but is predominantly tetraploid (Beams & King, 1942; Wilson & Leduc, 1948; Carriere, 1967).

Binucleate and multinucleate cells are commonly formed by failure of cytokinesis during mitosis or by fusion of the partially separated daughter cells. This mechanism was described for binucleate cells by Beams & King (1942) in a study of regenerating rat liver following partial hepatectomy. They also found that binucleate cells can themselves undergo mitosis, and that then both nuclei enter prophase together and the chromosomes combine on a single, large metaphase plate. If cytokinesis occurs, two large tetraploid cells are formed, but if not, a large binucleate cell with two tetraploid nuclei results. Extension of the process leads to the formation of cells of yet higher ploidy.

Wilson & Leduc (1948) and Nadal & Zajdela (1966) agreed that polyploid cells are formed in this way in the liver, but Wilson & Leduc (1948) proposed that endomitosis may be an additional means of change to higher ploidy, and also that cell fusion is probably involved in the formation of multinucleate cells, and nuclear fusion in the formation of polyploid nuclei.

From observations on cultures of mouse fibroblasts and spleen reticular cells, Fell & Hughes (1949) also observed the formation of binucleate cells by fusion of incompletely separated daughter cells and confirmed that they undergo the type of mitosis described by Beams & King (1942). However, the frequency of the process was too low to account for all the binucleate cells present and, although they did not obtain direct evidence, they also suspected that very large nuclei are formed by nuclear fusion. In cultures of the neoplastic HeLa cells several investigators have confirmed that when binucleate cells divide both nuclei enter prophase together, and they have also observed that all nuclei of multinucleate cells enter mitosis together, with the formation of complex metaphase figures. Following division, fusion of partially or even completely separated daughter cells results in cell groups of variable nuclearity (Oftebro & Wolf, 1967; Oftebro, 1968; Bednář, Fakan & Kašpar, 1968).

None of the above investigators obtained evidence of amitosis; in fact, Wilson & Leduc (1948) considered that the appearance of constricted and dumb-bell-shaped nuclei was more likely to indicate nuclear fusion or separation after fusion than an amitotic process. Nevertheless, a few authors still claim that amitosis is involved in binucleate cell formation. For example, Nagata (1962) found 11 labelled binucleate cells in the livers of rats sacrificed in groups at 8 and 24 hours after a pulse label with ^{3}H-T, and of these, 10 showed the label in only one nucleus. He concluded that this was most likely to be due to amitotic nuclear division in which all the newly synthesized DNA had been passed to one of the resulting nuclei.

The results of the present investigation indicate that, in transitional epithelium, cell fusion is the mechanism by which the large pyriform and surface cells are formed and that it normally occurs, not as an immediate sequel to mitosis, but at a later stage when cells are undergoing migration from the basal layer. Morphological appearances suggestive of cell fusion were occasionally seen in routine preparations,

but more substantial evidence was obtained from the manner of labelling of the binucleate cells in the long-term experiments. Some of these cells became labelled 11 weeks after the ^{3}H-T injection and in the majority only one nucleus was labelled, whilst in the remainder some were equally and other unequally labelled. It is difficult to conceive how this could occur other than by fusion of adjacent cells. The cell migration was so irregular that by the later time periods most labelled cells lay adjacent to unlabelled cells, which could account for the high frequency of binucleate cells with only one nucleus labelled.

There is also evidence that the large nuclei of the upper pyriform and surface cells which, like those of liver cells, often show irregular, constricted and dumb-bell shapes, are formed by nuclear fusion. Some of these nuclei showed a very much greater label than the maximum observed in the originally labelled basal cells, and the latter were the only source of nuclear label throughout the experimental period.

It is concluded that, in the guinea-pig, cell replacement and differentiation in transitional epithelium proceeds at a very slow rate in the following manner. Cells from the basal layer, which in this species is the progenitor layer, elongate to form small pyriform cells, and two or more of these fuse to form a large, upper pyriform cell. Following the fusion, the nuclei either remain separate, resulting in a binucleate pyriform cell, or they fuse to form a large, polyploid nucleus. A large, upper pyriform cell may enter the surface layer directly and expand over the summits of subjacent pyriform cells, or it may undergo fusion with one or more of its neighbours to form a very large bi- or multinucleate surface cell. The balance of nuclear to cytoplasmic material is thus maintained at all stages. During the 'migration' of cells towards the surface they retain their connexions with the basement membrane, but these connexions become drawn out into fine cytoplasmic processes or 'tails', which gives to the pyriform cell an appearance resembling a tadpole. The very large surface cells possess several cytoplasmic processes, which is itself suggestive of an origin from two or more cells. As Petry & Amon (1966) have stated, the fine cytoplasmic processes pursue a tortuous course between the underlying cells, and are undoubtedly an important factor in relocating the cells following their displacement during bladder distension.

The conclusions presented above have been supported by a supplementary study on the guinea-pig urinary bladder, and some of the results have been briefly reported (Martin, 1971). It was found that artificial ulceration of the mucosa provoked a greater mitotic response 24 hours after operation than did acute bladder distension, and that an enormous number of basal cells became labelled with ^{3}H-T. A special feature of the response, not found in the distension studies, was that for a brief period during the phase of marked cell proliferation some very large cells with gigantic nuclei appeared in the basal layer. Such cells are not a normal component of that layer, and they were apparently formed by cell fusion. Some of these cells underwent mitosis within the basal layer, with the formation of very large metaphase figures. It is hoped that the results of this supplementary study will form the basis of a later communication.

SUMMARY

Following acute distension of the guinea-pig urinary bladder, mitotic figures appeared in the basal layer of the epithelium 24 hours later, and at that time the labelling index with [^{3}H]thymidine was approximately 16 times the control level. The excess cell production caused areas of thickening and disorganization of the epithelium with cell shedding. Some of the labelled basal cells rapidly differentiated into upper pyriform and surface cells, but these were among those shed. The epithelium became quiescent and structurally normal within 10 days, yet contained a large number of well labelled cells, confined to the basal and lower pyriform cell layers. Thus, by killing animals after long periods following operation and ^{3}H-T injection the later history of this large pool of cells could be followed.

The cells slowly 'migrated' towards the surface in an irregular pattern and a few reached the surface layer after 11 weeks. Only those which remained in the basal layer for 6 weeks redivided. There was evidence that the large upper pyriform and surface cells were formed by fusion of adjacent cells during the migration. The nuclei sometimes remained separate and sometimes underwent fusion to form a large, polyploid nucleus. The cells maintained anchorage to the basement membrane by fine cytoplasmic 'tails' which are probably important in relocating the cells following their displacement during bladder distension.

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