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CELL PROLIFERATION IN THE EPITHELIUM OF THE OESOPHAGUS, TRACHEA AND URETER IN MICE

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SUMMARY

Over a 24-h period, groups of mice were given a single injection of colchicine (to collect blocked metaphases) and tritiated thymidine (to label nuclei synthesizing deoxyribonucleic acid). Epithelial nuclei in the oesophagus, trachea and ureter were examined and counted in paraffin sections: the duration of deoxyribonucleic acid synthesis (T_S) was calculated from the numbers of blocked metaphases and labelled nuclei, the duration of the post-synthetic gap (T_{G_2}) was estimated from the proportion of blocked metaphases labelled, and the cell cycle time (Tc) was calculated from T_S and the proportion of nuclei labelled. In each epithelium the different layers seen by light microscopy were analysed separately.

T_S was probably the same for the basal and superficial cells in the trachea (about 8 h), and was probably the same for the basal, intermediate and superficial cells in the ureter (about 5 h). In the oesophagus T_S was 8·5 h.

T_{G_2} was probably the same for the basal and superficial cells in the trachea (3·6 h), and probably the same for the basal, intermediate and superficial cells in the ureter (about 4·6 h). In the oesophagus T_{G_2} was 2·8 h.

Tc was about 380 h (basal cells) and 1400 h (superficial cells) in the trachea, and about 8000 h (basal and intermediate cells) and 2700 h (superficial cells) in the ureter. In the oesophagus Tc was 41 h.

INTRODUCTION

Combined injection of colchicine (to collect blocked metaphases) and tritiated thymidine (to label nuclei synthesizing deoxyribonucleic acid) has previously been used to investigate the proliferation of stratified squamous epithelium in mice (Blenkinsopp, 1968*a*). The present report describes the results obtained with an improved technique: mice were again used, the oesophageal epithelium was examined for comparison with previous results, and the tracheal and ureteric epithelia were studied.

Previous study of the mouse oesophagus showed that following intraperitoneal injection of colchicine accumulation of blocked metaphases was linear up to 5 h after injection (Blenkinsopp, 1968*a*): the mice were therefore killed 4·8 h after injection in the present experiment.

The results were used to obtain the duration of deoxyribonucleic acid synthesis (T_S), the post-synthetic gap (T_{G_2}), and the cell cycle time (Tc).

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MATERIAL AND METHODS

Twenty male inbred *C3H/He* mice (age 8 weeks, weight 24 g) divided into 5 groups of 4 mice, were each given a single intraperitoneal injection of colchicine (2 mg/kg body weight) and tritiated thymidine (1·5 μCi/g body weight, specific activity 5·0 Ci/mM), and killed 4·8 h after the injection. One group was killed every 4·8 h for 24 h, and at sacrifice the oesophagus, trachea and both ureters were removed from each animal, fixed in Bouin's solution, and processed for histology. Transverse paraffin sections (5 μm) were cut from the oesophagus and trachea just below the level of the thyroid gland and from the ureters at about their mid-point, and stained with periodic acid/Schiff; autoradiographs were prepared using Ilford K5 nuclear emulsion (exposed for 4 weeks at 0–4 °C), and the sections were finally stained with Mayer's haemalum. Epithelial nuclei were counted (about 6000 oesophageal, 3000 tracheal and 4000 ureteric nuclei per mouse). These were divided into basal (the single basal layer) and superficial (the remainder) in the trachea and oesophagus, as described in previous papers (Blenkinsopp, 1967*a*, 1968*a*). In the ureters they were divided into basal (the single layer resting on the basement membrane), superficial (the single layer of large cells with large nuclei which surrounds the lumen), and intermediate (the remainder). The count in each layer of cells was subdivided into total labelled nuclei, total metaphases, labelled metaphases, and total nuclei present. Nuclei were considered labelled if they were overlaid by 4 or more grains.

For comparison with the counts on tracheal epithelium, similar counts were made on sections of trachea from mice used in a previous experiment (Blenkinsopp, 1968*a*), where the materials and methods were exactly the same except that only 4 groups of mice were used, the mice were killed 6 h after injection, and the data required correction for loss of blocked metaphases during the final hour before sacrifice.

RESULTS

General observations

Probably only the basal cells proliferate in the oesophageal epithelium (Marques-Pereira & Leblond, 1965) and thus labelled 'superficial' nuclei represent a population of basal cells which are apparently superficial because of oblique cutting; the appropriate correction was described in a previous paper (Blenkinsopp, 1968*a*), and was used in the present analysis of oesophageal epithelium. The corrected counts are given in Table 1. This correction is not required for the tracheal and ureteric data because the layers of cells can be more readily distinguished, particularly when the periodic acid/Schiff reaction is used.

The results of counts on the tracheal epithelium are set out in Table 2; in order to simplify this Table, the separate values for basal and superficial metaphases (labelled

Table 1. *Mean values (± standard deviation) for number of labelled basal nuclei (including labelled metaphases), total metaphases (including labelled metaphases), labelled metaphases, and total superficial nuclei, per* 100 *basal nuclei in mouse oesophagus*

	Time of injection (G.M.T.)					
	23.24	04.12	09.00	13.48	18.36	Mean
Labelled basal nuclei	17·6 ± 4·8	18·3 ± 2·6	21·5 ± 4·2	10·7 ± 4·8	7·2 ± 1·8	15·1
Total metaphases	5·30 ± 2·5	9·94 ± 2·3	14·25 ± 4·1	6·64 ± 3·4	4·02 ± 1·2	8·03
Labelled metaphases	1·86 ± 0·3	3·78 ± 0·2	7·13 ± 0·7	2·94 ± 0·3	1·30 ± 0·0	3·40
Total superficial nuclei	47 ± 9	40 ± 6	64 ± 15	68 ± 14	67 ± 7	57

and total) are not given in the Table but are given in the text where appropriate. Nuclei were seen on the top of the tracheal epithelium, apparently resting on it (mean 14, standard deviation 8, per 100 basal nuclei); some were macrophages, none were labelled or in mitosis, and the conclusion was reached that they were not living epithelial nuclei: they are therefore not included in the Table or the subsequent analysis.

The counts of metaphases and labelled nuclei were very low in the ureteric epithelium, and the actual counts are given in Table 3.

Table 2. *Mean values (* ± *standard deviation) for number of labelled basal nuclei (including labelled basal metaphases), labelled superficial nuclei (including labelled superficial metaphases), total metaphases (including labelled metaphases), labelled metaphases, and total superficial nuclei, per* 100 *basal nuclei in mouse trachea*

	Time of injection (G.M.T.)					
	23.24	04.12	09.00	13.48	18.36	Mean
Labelled basal nuclei	1·77 ± 0·5	1·28 ± 0·3	2·66 ± 1·6	1·22 ± 0·6	0·79 ± 0·3	1·54
Labelled superficial nuclei	0·65 ± 0·3	0·39 ± 0·3	1·09 ± 1·3	0·60 ± 0·5	0·22 ± 0·1	0·59
Total metaphases	1·49 ± 0·5	1·39 ± 0·8	3·30 ± 3·6	1·91 ± 0·4	1·05 ± 0·4	1·83
Labelled metaphases	0·49 ± 0·1	0·24 ± 0·1	1·09 ± 1·4	0·34 ± 0·2	0·18 ± 0·2	0·47
Total superficial nuclei	124 ± 17	112 ± 7	123 ± 44	122 ± 43	145 ± 11	125

Previous experiment

	Time of injection (G.M.T.)				
	17.00	23.00	05.00	11.00	Mean
Labelled basal nuclei	1·30 ± 1·1	1·05 ± 1·3	1·42 ± 0·5	2·55 ± 1·3	1·58
Labelled superficial nuclei	0 ± 0	0·32 ± 0·6	0·65 ± 0·5	0·62 ± 0·5	0·40
Total metaphases	0·94 ± 0·7	0·56 ± 0·3	1·03 ± 0·4	1·65 ± 0·5	1·045
Labelled metaphases			Not counted		
Total superficial nuclei	112 ± 27	116 ± 41	105 ± 18	117 ± 51	113

Determination of T_S

Colchicine acts at the beginning of metaphase (Ham, 1965; Taylor, 1965); thus when colchicine is given there is a delay (the duration of metaphase) before metaphases apparently begin to accumulate. However, if the accumulation of blocked metaphases is linear, and if colchicine begins to act immediately after injection, then metaphases plotted against time after injection of colchicine should give a straight line going through 0 metaphases at 0 time. The data of Tannock (1967, fig. 2), Dustin (1959, fig. 1), Bertalanffy & Leblond (1953, fig. 1) and Blenkinsopp (1968*a*, table 1) all permit this interpretation. This suggests that the number of metaphases found, for example, 4·8 h after injection of colchicine is in fact the number of nuclei which have entered mitosis in 4·8 h.

Table 3. *Actual counts of labelled nuclei (including labelled metaphases), total metaphases (including labelled metaphases), labelled metaphases, and total number of nuclei, in the basal, intermediate and superficial layers of cells in mouse ureter*

	Time of injection (G.M.T.)					
	23.24	04.12	09.00	13.48	18.36	Total
Basal						
Labelled nuclei	3	0	6	5	3	17
Total metaphases	4	2	6	8	1	21
Labelled metaphases	1	0	0	1	0	2
Total nuclei	7520	6960	7230	6560	8130	36400
Intermediate						
Labelled nuclei	0	1	10	7	1	19
Total metaphases	1	4	6	1	2	14
Labelled metaphases	0	0	0	0	0	0
Total nuclei	8970	8130	8240	7960	8100	41400
Superficial						
Labelled nuclei	1	0	5	2	0	8
Total metaphases	1	0	3	0	0	4
Labelled metaphases	0	0	0	0	0	0
Total nuclei	1250	1220	1090	1060	1190	5810

The effective labelling time (v hours) of tritiated thymidine following intraperitoneal injection is about 0·5 h (Blenkinsopp, 1968*b*; Bresciani, 1965; Rubini, Cronkite, Bond & Fliedner, 1960; Skougaard & Stewart, 1966). Then

$$\frac{\text{mean number of labelled nuclei}}{\text{mean number of blocked metaphases}} = \frac{T_S+v}{4{\cdot}8}.$$

From the present data, T_S for oesophageal cells was 8·5 h.

The impression was gained from previous studies that nuclei in mitosis tended to become displaced superficially, as observed by Tannock (1967) in the intestinal crypts, and in the present study of tracheal epithelium comparison of the basal: superficial ratio for metaphases (0·82:1·01 per 100 basal nuclei = 0·82) with that for labelled nuclei (1·54:0·59 per 100/basal nuclei = 2·61) strongly suggested that this displacement caused some of the basal metaphases to be wrongly classified as superficial. T_S for the basal cells alone was 8·5 h, and for the superficial cells alone T_S was 2·3 h. In view of these results it seems probable that T_S was the same for both basal and superficial cells. Using the data for total metaphases and total labelled nuclei in the trachea, T_S was 5·1 h in the present experiment, and 10·9 h in the previous experiment (using a similar technique for the calculation): the mean of these two values was 8·0 h.

In the ureteric epithelium, using the data for total metaphases and total labelled nuclei, T_S was 4·9 h. The small number of these cells renders subdivision of doubtful validity, but if the layers were considered separately T_S was 3·4 h for the basal cells, 6·0 h for the intermediate cells, and 9·1 h for the superficial cells. As in the tracheal

epithelium, it seems probable that T_S was the same for all layers of the ureteric epithelium (4·9 h).

Determination of T_{G2}

$$\frac{\text{Unlabelled metaphases}}{\text{Total metaphases}} = \frac{T_{G2}}{4{\cdot}8}.$$

In the oesophageal epithelium T_{G2} was 2·8 h.

The tracheal data from the previous experiment are of no value for this calculation because of loss of blocked metaphases towards the end of the 6-h period between injection of colchicine and sacrifice of the animals (Blenkinsopp, 1968*a*). In the present experiment there were 0·25 labelled and 0·82 total basal metaphases, and 0·22 labelled and 1·01 total superficial metaphases, per 100 total basal cells: thus T_{G2} was 3·6 h (basal and superficial cells combined), or 3·3 h (basal cells alone) and 3·8 h (superficial cells alone). It seems probable that T_{G2} was the same for both basal and superficial cells (3·6 h).

In the ureteric epithelium, T_{G2} was 4·6 h (combined data), or 4·4 h (basal cells) and 4·8 h or more (intermediate and superficial cells). As in the tracheal epithelium, it seems likely that T_{G2} was the same for all layers of the ureteric epithelium (4·6 h).

Determination of Tc

Cells are randomly lost from the basal layer in the oesophageal epithelium (Marques-Pereira & Leblond, 1965), and in the present work the assumption has been made that cell loss is random from each layer of all the epithelia studied. In this event, cells in each proliferative layer are distributed exponentially through the cell cycle (Brown & Oliver, 1968). Because of the probability already referred to of basal metaphases being displaced superficially, *Tc* has been calculated not from the most direct formula,

$$\frac{\text{total metaphases}}{\text{total nuclei}} = \frac{4{\cdot}8}{Tc}\log_{e}2,$$

but from the similar formula for labelled nuclei:

$$\frac{\text{labelled nuclei}}{\text{total nuclei}} = \frac{T_S+v}{Tc}\log_{e}2,$$

where v is the duration of availability of tritiated thymidine following intraperitoneal injection (0·5 h).

In the oesophageal epithelium *Tc* was 41 h.

The mean values from the present and previous experiments on the tracheal epithelium have been used to calculate *Tc*, which was 380 h for the basal cells and 1400 h for the superficial cells.

In the ureteric epithelium, *Tc* was 8000 h for the basal cells, 8200 h for the intermediate cells, and 2700 h for the superficial cells.

DISCUSSION

T_S

The calculations in the present work depend on the assumption that all the cells in each proliferative layer have the same Tc, T_S and T_{G2}; this has not been established, but the available evidence suggests that only in some epithelia do a small proportion of cells differ from the rest (Cameron & Cleffmann, 1964; Gelfant, 1966). Wolfsberg (1964) suggested that a large proportion of the basal cells in the forestomach epithelium differed from the rest, but this conclusion was based on results which ignored diurnal variation: the conclusion is therefore invalid.

There is adequate evidence that the doses of colchicine and tritiated thymidine used were appropriate (Blenkinsopp, 1967*b*, 1968*a*), and that the availability time of tritiated thymidine following intraperitoneal injection is about 0·5 h (Blenkinsopp, 1968*b*; Bresciani, 1965; Rubini *et al.* 1960; Skougaard & Stewart, 1966).

In an earlier experiment (Blenkinsopp, 1968*a*) T_S was 9·1 h in mouse oesophagus (the availability time of tritiated thymidine should be subtracted from this, giving 8·6 h), and the range in rats and mice reviewed in that paper was 4·8–8·6 h in the oesophagus and 7–10 h in the tongue. In the mouse forestomach T_S was 13·5 h (Wolfsberg, 1964), 7 h (Frankfurt, 1967), and 8·4 or 6·9+ h (Pilgrim, 1964); in the rat forestomach T_S was 9·6 or 7·2 h (Pilgrim, 1964). In the present experiment T_S was 8·5 h in mouse oesophagus, and these figures suggest that in the oral, oesophageal and forestomach-epithelium of mice and rats T_S is 7–9 h. Bresciani (1965) has pointed out that a variety of different ways have been used to analyse the curve of labelled mitoses obtained after single injection of tritiated thymidine, and that the duration of the pulse of tritiated thymidine has often been ignored: these factors account for at least some of the differences between reported values for T_S.

Wegener, Hollweg & Maurer (1964) found that in foetal rat bronchial epithelium T_S was 5·0 h (and in the oesophageal epithelium T_S was 5·1 h). Shorter, Titus & Divertie (1966) gave adult rats single injections of tritiated thymidine and found that the percentage of mitotic figures labelled was none at 1 h, approximately 25% at 2 h, 50% at 4 h, 100% at 6 h, and 25% at 12 h: on this evidence T_S appears to be about 8 h. The present work suggests that T_S is the same for the basal and superficial cells in tracheal epithelium, and is about 8 h.

No previous report of the estimation of T_S in urinary tract epithelium has been found. The present work suggests that T_S is the same for the basal, intermediate and superficial cells, but the value obtained for this (4·9 h) must be considered a very approximate estimate because of the technical difficulties involved in studying cells with a very low rate of proliferation.

T_{G2}

Messier & Leblond (1960) found that in most tissues T_{G2} was more than 1 h, and Koburg & Maurer (1962) gave a range in various tissues of 30 min to 16 h. In mouse oesophageal epithelium Pilgrim & Maurer (1965) found that $T_{G2}+T_M$ (duration of mitosis) was 5–6 h, and from Cameron & Greulich (1963, fig. 2) $T_{G2}+\frac{1}{2}T_M$ appears

to be 2·5 h. In mouse tongue $T_{G2}+\frac{1}{2}T_M$ appears to be 3 h (Cameron, 1966, fig. 12). In the forestomach epithelium of the mouse T_{G2} was 1–2 h (Wolfsberg, 1964) and 3·5 h (Frankfurt, 1967). In the hamster cheek pouch $T_{G2}+\frac{1}{2}T_M$ was 2·4 h (Brown & Oliver, 1968). In the present work T_{G2} was 2·8 h for oesophageal epithelium.

Shorter *et al.* (1966) gave 4 points for labelled mitoses in tracheal epithelium after a single injection of tritiated thymidine, from which $T_{G2}+\frac{1}{2}T_M$ appears to be 3·5 h. The present work suggests that T_{G2} is the same for basal and superficial cells in the mouse trachea, and is about 3·6 h.

There is no previous report of T_{G2} in urinary tract epithelium, and the present results give only a rough approximation; however, T_{G2} appears to be the same in basal, intermediate and superficial cells, and this value appears to be about 4·6 h.

Tc

The estimation of *Tc* from two waves of labelled mitoses after a single injection of tritiated thymidine assumes that all proliferating cells have the same *Tc*, but is the only method so far available which is not affected by the presence of cells which are not proliferating. This technique has been used on mouse forestomach, where *Tc* was 30 h (Wolfsberg, 1964) and 28 h (Frankfurt, 1967), and on the tongue and palate epithelium of mice, where *Tc* was about 97 h (Dhawan & Toto, 1965). Cameron (1966) calculated that *Tc* in the tongue epithelium was 21–35 h, from T_S and the number of labelled nuclei after a single injection of tritiated thymidine. The number of mitoses blocked by colchicine was used in a previous experiment to calculate *Tc*, which was 58 h in mouse oesophagus, 53 h in the ventral surface of the tongue, and 34 h in the dorsal surface of the tongue (Blenkinsopp, 1968*a*). In the present work, the same principle was used with better technical details, and *Tc* was 41 h in oesophageal epithelium.

No valid assessment of the cell cycle time in respiratory tract epithelium has so far been published, and much of the work in this field was discussed in a previous paper (Blenkinsopp, 1967*a*). The nearest approach is that of Bertalanffy & Lau (1962), who found that 2·1 cells per 100 total cells were blocked in metaphase in rat tracheal epithelium per 24 h: if the ratio of basal to superficial cells in rats is 100:253 (Blenkinsopp, 1967*a*), this is equivalent to 7·4 metaphases per 100 basal cells per 24 h. It is of interest to note that this value falls between the two present values (4·2 and 9·1, from Table 2). In the present work *Tc* was about 380 h for the basal cells and about 1400 h for the superficial cells. However, this estimate of *Tc* for the superficial cells is likely to be an oversimplification, as there appears to be more than one population of cells in this layer (Blenkinsopp, 1967*a*; Bindreiter, Schuppler & Stockinger, 1968).

Leblond, Vulpe & Bertalanffy (1955) studied the bladder epithelium in adult rats, using colchicine, and found that 3·1% of the superficial cells and 1·6% of the deep cells were blocked in metaphase in 24 h, i.e. *Tc* was 540 h for the superficial cells and 1040 h for the deep cells. These figures are about one-fifth of those found in the present study of ureteric epithelium. Bullough (1946) studied different parts of the urinary tract in female mice given colchicine, and his figures can be used for comparison of one part with another. Bullough (1946) found that the epithelium of the

trigone and urethra resembled stratified squamous epithelium and cell divisions were most numerous in the basal layer, whereas in the ureter and the bladder except the trigone the epithelium was clearly transitional and cell divisions were most numerous in the superficial layer. The present author has added together the figures given by Bullough for the different phases of the oestrous cycle, so as to produce a rough estimate of the comparative frequency of blocked metaphases in the different epithelia; the sums are: ureter 0·24, bladder except trigone 0·05, trigone and urethra 7·50, and oesophagus 36·9. Then, if 36·9 blocked metaphases in the oesophagus (Bullough, 1946) is equivalent to a *Tc* of 41 h (present work), *Tc* for the basal cells of the trigone and urethra is about 200 h, and the mean *Tc* for all the cells of the ureteric epithelium is about 6300 h; for comparison with this value derived from Bullough, the mean *Tc* for the cells of the ureteric epithelium in the present work is about 7100 h. The author appreciates the speculative nature of these calculations, but considers them worthy of mention because there are very few reports of work on these very slowly proliferating cells. The present work indicates that *Tc* is probably the same for basal and intermediate cells in the ureter (about 8000 h) but is different for superficial cells (about 2700 h), and Bullough's (1946) work suggests that in the bladder epithelium excluding the trigone the cell cycle times are probably about 5 times as long.

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