

CELL KINETICS OF URETHANE INDUCED MURINE PULMONARY ADENOMATA:

I. THE GROWTH RATE

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Summary.—A single injection of urethane into adult male A2G mice produced an increase in the proliferative rate of alveolar wall cells, reaching a peak at 2 weeks post urethane (PU) and declining to control levels by 2 months PU. During this urethane induced proliferative response the single and double labelling indices and the native metaphase index were all elevated although there was no corresponding alteration in the arrested metaphase index. This proliferative response may not be restricted to hyperplasia of potentially neoplastic cells, such as type II epithelium, but may also include type I epithelial cells and alveolar macrophage precursors. However, it was impossible to identify individual cell populations by the methods used.

The growth rate of adenomata decreased with time and cell kinetic techniques showed that the rates of entry of adenoma cells into DNA synthesis and into metaphase were decreasing concurrently with the growth rate. Thus the rate of cell production falls as adenomata age but how much cell loss contributes to the decrease in growth rate is not yet known. Decreasing cell production could be due to an increased cell cycle time and/or a decreased growth fraction. The duration of DNA synthesis in adenomata increased markedly as the mice survived, suggesting that the cell cycle time might be increased, but further experiments are required to determine whether the growth fraction changes.

Attention is drawn to a complication that Colcemid introduces into kinetic studies on alveolar wall cells.

THE DISCOVERY by Nettleship, Henshaw and Meyer (1943) that urethane (ethyl carbamate) treatment increased the incidence of pulmonary adenomata in A strain mice prompted its use as a model of tumorigenesis by many investigators, but most studies merely relied on enumeration of surface adenomata. Rogers (1951) took into consideration both the number and size of these superficial adenomata. In addition, Shimkin and Polissar (1955) monitored the number and size of adenomata seen in serial sections of mouse lungs at various times after a single urethane injection. In this way they discovered that the growth of adenomata was "self-limiting", in that their rate of

growth decreased with age. Stewart (1959), however, regarded murine pulmonary adenomata as adenocarcinomata of low-grade malignancy but, although they were unencapsulated, metastasis was not a feature and they rarely proved fatal. To elucidate how the growth of adenomata is controlled a cell kinetic study of murine alveolar tissue has been carried out following urethane treatment.

The most important requisite in such a study is to construct a population growth curve of adenomata over a long period of their development. The growth of adenomata is the resultant between the rate of cell production by mitosis and the rate of cell loss by death. Any

change in growth rate with age may be explicable by changes in these two factors. Cell production rates can be estimated by stathmokinetic or tritiated thymidine ($^3\text{H-TdR}$) labelling techniques. Simple pulse labelling and metaphase arrest techniques yield only a limited amount of kinetic information, and hence stathmokinesis has been combined with double labelling using two comparable groups of mice. In one group metaphase arrest was followed by a single injection of $^3\text{H-TdR}$, whilst simultaneously the other group received $^3\text{H-TdR}$ plus a second injection of $^3\text{H-TdR}$ 1 h later. The double labelling index (I_{DL}) will be higher than the single labelling index (I_{S}) on account of cells entering DNA synthesis in the 1 h period, the difference ($I_{\text{DL}} - I_{\text{S}}$) being a measure of the rate of entry of cells into DNA synthesis per h (R_{S}). A knowledge of I_{S} and R_{S} allows the duration of DNA synthesis (t_{S}) to be estimated, since $t_{\text{S}} = I_{\text{S}}/R_{\text{S}}$. The rate of entry of cells into mitosis per h (R_{M}) can be calculated by dividing the arrested metaphase index ($I_{\text{M(a)}}$) by the duration of the arrest period, assuming that (a) cells already in metaphase at the time of treatment are not arrested, (b) the accumulation of arrested metaphases occurs in a linear manner and (c) significant numbers of arrested metaphases do not degenerate during the arrest period. The native metaphase index (I_{M}) may be derived from the double labelling group, and the duration of metaphase (t_{M}) is given by $t_{\text{M}} = I_{\text{M}}/R_{\text{M}}$. From R_{S} and R_{M} estimates of cell production (k_{B}) can be made, and the rate of cell loss (k_{L}) assessed from the difference between k_{B} and the observed growth rate (k_{G}) obtained by direct measurement of the tumours. Such computations assume a steady flow of cells through the cell cycle with no diurnal effects, that Colcemid (demecolcine) does not affect labelling, that all tumour growth is due to mitotic division and that all cells entering DNA synthesis subsequently enter mitosis. It is also assumed that

all adenomata begin to develop at the same time.

MATERIALS AND METHODS

Male specific pathogen-free (SPF) A2G mice were obtained in batches from the Laboratory Animals Centre, Carshalton, over a period of about 12 months. In order to minimize possible seasonal effects, all mice were kept in a room with controlled light and temperature. Furthermore, for the first 3 months of life animals were maintained under SPF conditions to minimize the possibility of incidental lung infection. At 3-4 months of age approximately 50% of any batch were given a single intraperitoneal (i.p.) injection of a 4% w/v solution of urethane (BDH) in a volume of saline adjusted to give a dose of 1 mg/g body weight. The remaining mice served as controls and were injected with an equivalent volume of normal saline. The mice survived for 0, 2, 4, 9, 17 and 26 weeks and were killed in groups of 20, 10 being experimental and 10 control. A comparable group of 10 experimental and 10 control female A2G mice were left for 26 weeks in order to check the influence of the sex hormonal environment on urethane tumorigenesis.

Having weighed the mice, the experimental and control groups were separated into subgroups of 5. One experimental and one control subgroup were injected i.p. at 10.30 h with a saline solution of Colcemid (Ciba) whose concentration was 1 mg/ml, the dose being 20 $\mu\text{g/g}$ body weight. At 13.00 h these mice received $^3\text{H-TdR}$ i.p. (specific activity 5 Ci/mmol, Radiochemical Centre, Amersham) at a dose of 0.5 $\mu\text{Ci/g}$ body weight, the concentration of the saline solution of $^3\text{H-TdR}$ being 40 $\mu\text{Ci/ml}$. The mice were killed at 14.30 h to give a maximum metaphase arrest of 4 h. The other experimental and control subgroups did not receive Colcemid but were given 2 $^3\text{H-TdR}$ injections, one at 13.00 h and the other at 14.00 h before killing at 14.30 h. To increase the precision of the growth curve of surface adenomata, it was necessary to interpolate comparable groups of 10 SPF derived male A2G mice at intervals of 7.5, 22.5 and 32.5 weeks post urethane (PU). All these mice received a single injection of $^3\text{H-TdR}$ 1.5 h before being

killed. In order to monitor the short-term reaction of the alveolar walls to the carcinogen, 2 further groups of 10 mice received stathmokinetic treatment and double thymidine labelling at 1 and 3 weeks PU.

Mice were killed by neck fracture, their lungs fixed in Carnoy's fluid for 24 h and then placed in Cellosolve for 2 weeks. In the left lung the number and size of adenomata visible on the surface were determined under a dissecting microscope ($\times 12.5$) containing a linear eyepiece graticule. An index of the size of the adenomata was obtained by measuring 2 horizontal diameters at right-angles and multiplying them to give an equatorial area index (A_E). The left lung was then embedded in paraffin and about twenty $5 \mu\text{m}$ serial sagittal sections taken. Alternate sections were used for autoradiography by stripping film and after 4 weeks' exposure stained with Harris' haematoxylin. To facilitate metaphase counting the remaining sections of the ribbon were stained by the periodic acid-Schiff (PAS) method.

A square eyepiece graticule, representing a field, was used to count metaphases and labelled nuclei of *alveolar wall cells*. Sections were traversed from apex to base until at least 100 fields (excluding large blood vessels, bronchioles and lymphoid aggregations) had been examined in both PAS stained sections and autoradiographs, to determine the total numbers of metaphases and labelled cells. All the nuclei, excluding those of alveolar macrophages, in every 20th field were counted to obtain the mean number per field, from which was derived the total number of alveolar wall cells scanned and the percentage metaphase and labelling indices.

The counting procedure was modified for determination of metaphase and labelling indices of *adenomata*. The number of whole graticule squares covering the adenoma was first determined at $\times 100$ and then the total metaphases were counted at $\times 1000$. From 10 fields taken at random, the mean nuclear count per field was obtained and hence, by utilizing the area magnification factor between $\times 100$ and $\times 1000$ magnifications, the total number of nuclei in the area of the adenoma was calculated. The procedure was repeated for the corresponding adenoma in the autoradiograph to determine the total labelled cells and total nuclei scanned.

Percentage labelling and metaphase indices were calculated as for the alveolar tissue.

These counting methods employed for adenomata and for alveolar tissue were dictated by the low proliferative rates and the necessity to include sufficient labelled cells or metaphases. The methods were considered to be adequate since our chief concerns were the relative differences between experimental and control groups and the relative changes with time rather than the absolute values.

RESULTS

Growth of surface adenomata

The mean number of surface adenomata per experimental left lung is plotted against time in Fig. 1. Adenomata first became visible 4 weeks after urethane and then increased in number rapidly and approximately linearly until about 16 weeks PU, when the curve flattened. The incidence of adenomata in control mice was only 0.14, indicating that the vast majority of adenomata seen in treated mice was attributable to the action of urethane.

The A_E of each surface adenoma was initially expressed in graticule units of area and plotted against time PU. However, in mice killed at any particular time the distribution of A_E was not normal but tended to conform to a positively skewed log-normal distribution. Using $\log A_E$ as ordinate and cumulative percentage of neoplasms (probit) as abscissa, a few groups did give approximately straight lines (Fig. 2), suggesting a log-normal distribution; other groups, however, failed to produce such lines and it was therefore decided to use the median A_E value (at the 50% point) as representative of each group.

To construct an overall growth curve for the surface adenomata, the median A_E of each group was converted into an estimate of volume by assuming that the adenomata were spherical, this being justified by their shape macroscopically and on section. The log median adenoma volume plotted against time (Fig. 3) gave a curve suggesting a power relation-

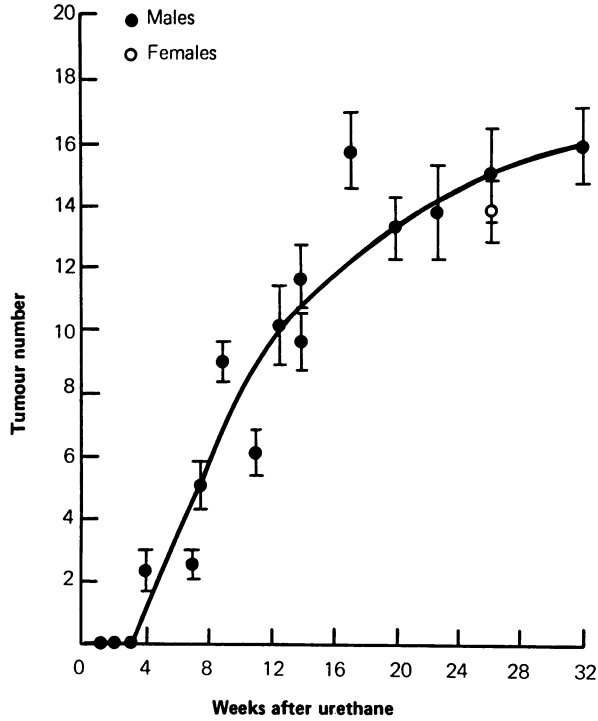


FIG. 1.—Relation of surface tumour number and survival.

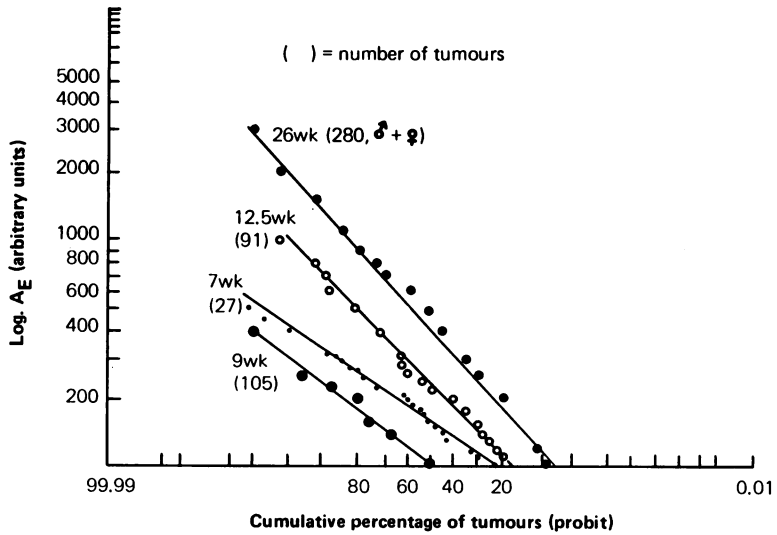


FIG. 2.—Tumour area distribution in relation to survival.

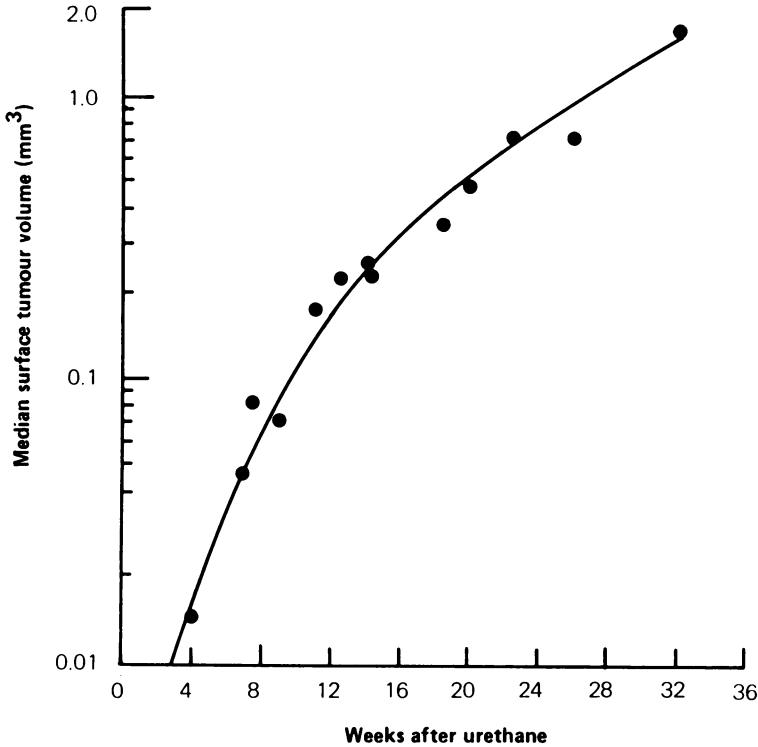


Fig. 3.—Relation of tumour volume and survival (log-linear).

ship, and log of median volume plotted against the log of time gave a straight line, which was fitted to the data by least squares linear regression analysis. The coefficients of regression were next used to derive an equation describing the growth of the surface adenomata from 4 to 32.5 weeks PU:

$$\log_{10} V = (2.181 \times \log_{10} t) - 3.11$$

where V = median volume of surface adenomata in mm^3 and t = time in weeks PU. This equation can be simplified to:

$$V = 0.000776 \times t^{2.181}$$

The specific growth rate (k_G) of the adenomata, defined as the growth rate per unit volume,

$$\frac{1}{V} \cdot \frac{dV}{dt}$$

is given by differentiating $\log V$ with respect to t :

$$\begin{aligned} k_G &= \frac{d(\log V)}{dt} \\ &= \frac{1}{V} \cdot \frac{dV}{dt} \\ &= \frac{2.181 \times 0.000776 t^{1.181}}{0.000776 t^{2.181}} \end{aligned}$$

i.e.

$$k_G = \frac{2.181}{t}$$

This equation shows that the growth rate of the adenomata decreases with time.

Adenoma metaphase and labelling indices

Figure 4 shows that both the native metaphase index (I_M) and the 4 h Col-

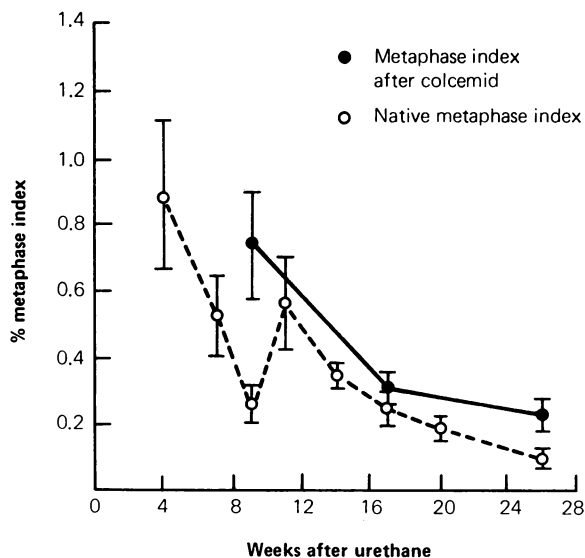


FIG. 4.—Metaphase index of adenomata in relation to survival.

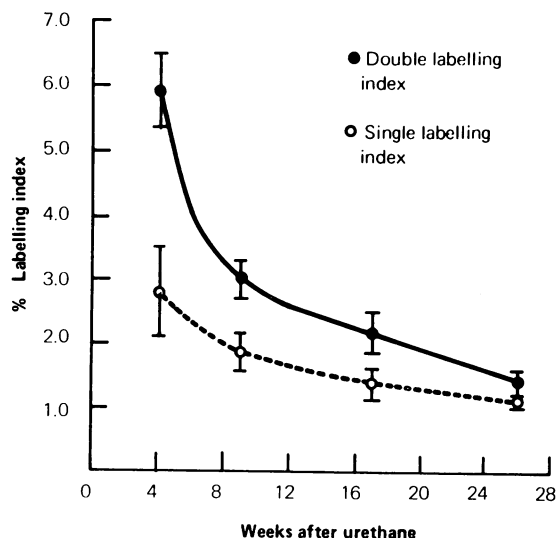


FIG. 5.—Labelling index of adenomata in relation to survival.

cemid metaphase index ($I_{M(a)}$) decrease with increasing age of the adenomata. Since $I_{M(a)}$ is an index of the rate of entry into metaphase (R_M), the birth rate (k_B) or rate of adenomata cell production is also decreasing. Similarly, I_S and I_{DL} fall with time (Fig. 5). The difference between the two curves

($I_{DL} - I_S$) likewise decreases, indicating that R_S falls with age. The Table gives the values of R_M , R_S , I_M and I_S as well as the calculated values of t_M and t_S .

Female mice

There were no significant differences between adenomata occurring in males

and females at 26 weeks PU. The mean number of surface adenomata per left lung in urethane treated mice was 15 for males and 14 for females. The median sizes of the surface adenomata in both sexes were similar. The pro-

liferative rates of the adenomata as shown by the R_S and R_M values were slightly higher in females than in males, but these differences were not significant. The only difference between the sexes was in their weights at death, the average

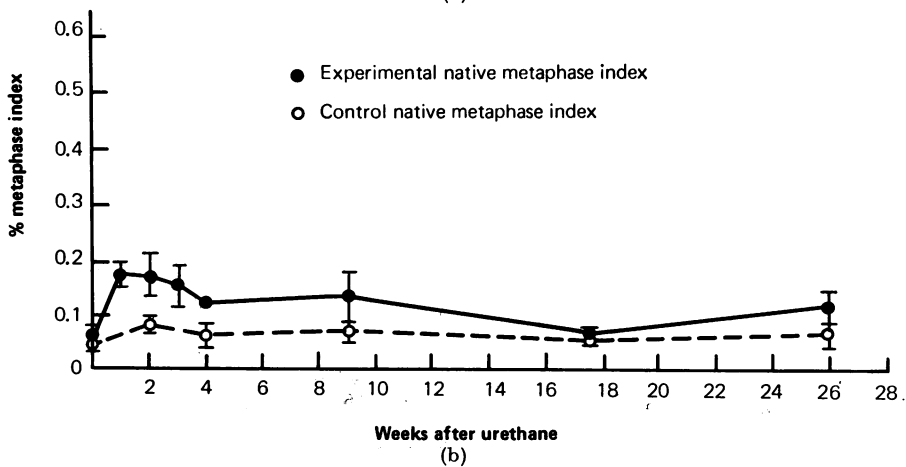
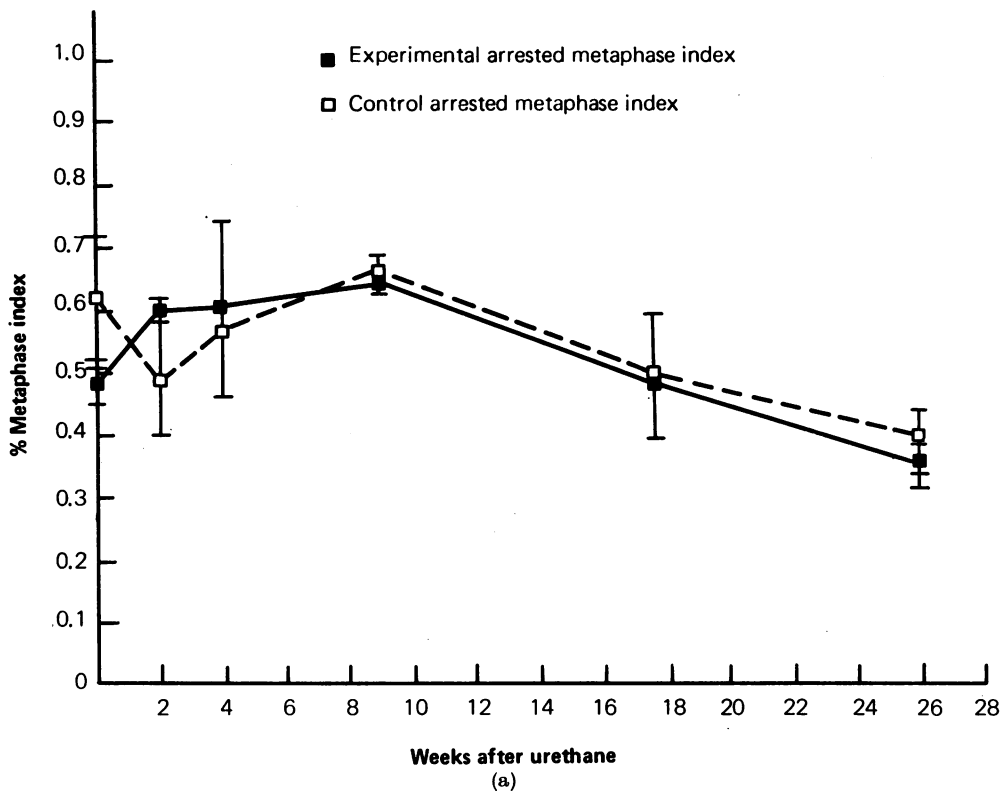


FIG. 6.—Metaphase index of alveolar tissue in relation to survival: (a) arrested metaphases; (b) native metaphases.

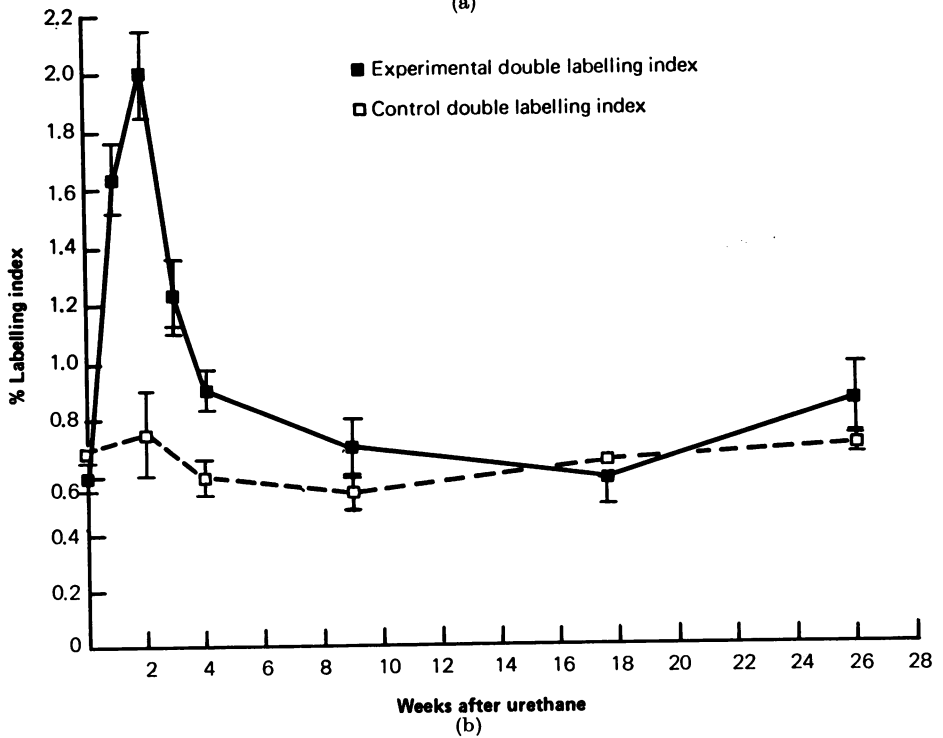
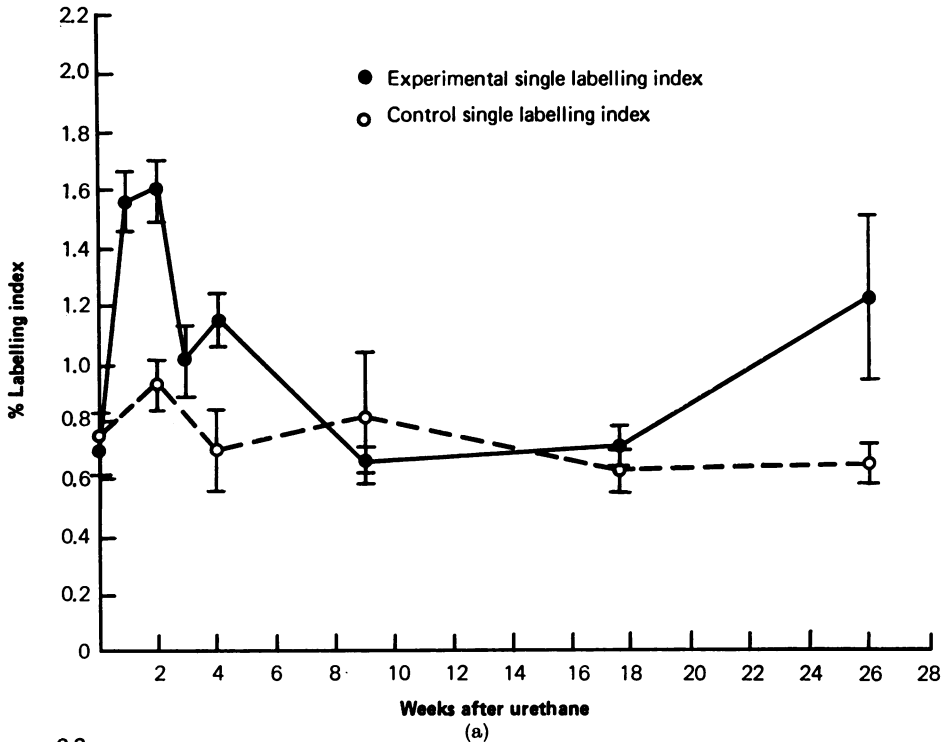


FIG. 7.—Labelling index of alveolar tissue in relation to survival: (a) single indices; (b) double indices.

weight of females being 34 g compared with 41 g for males, a difference probably due to the larger fat deposits in males. These observations support the conclusions of Shimkin (1955) that urethane induced adenomata are not influenced by the sex hormonal environment. All the cell kinetic data from males and females were accordingly combined for the 26-week interval.

Alveolar tissue

Figure 6 shows no significant differences between $I_{M(a)}$ in experimental and control mice. However, there is a urethane effect on I_M since the experimental values are all higher than those of the corresponding controls. The experimental I_M values also show a peak response at 2-weeks PU whereas the control values remain approximately constant with time.

The labelling indices (Fig. 7) reveal increased proliferative activity in the first month after urethane treatment. Both I_S and I_{DL} in the experimental mice have peak values at 2 weeks PU but return to control levels by 2 months PU. However, the labelling indices exhibit an anomaly, in that I_S obtained from Colcemid treated mice is on the

whole higher than I_{DL} . This phenomenon is evident in control mice at 0, 2, 4 and 9 weeks and in experimental ones at 4, 17 and 26 weeks PU. Only in the experimental peak values at 1, 2 and 3 weeks PU is I_{DL} larger than I_S , as might be expected throughout. At the 26-week interval no differences were evident between males and females for any of the alveolar tissue parameters, and the values were therefore combined into a single group.

DISCUSSION

Adenomata

The urethane induced surface adenomata increase in size in such a way that their growth from 4 to 32.5 weeks PU conforms, as already indicated, to a quadratic equation: $V=0.000776 \times t^{2.181}$. However, with such a simple growth equation extrapolation beyond the limits of 4 and 32.5 weeks is not justified. Surface adenomata display a decrease in growth rate as they become older. The change in growth rate could be due to a decrease in k_B and/or an increase in k_L but no areas of necrosis were apparent throughout the whole observation period. The decreases in R_M

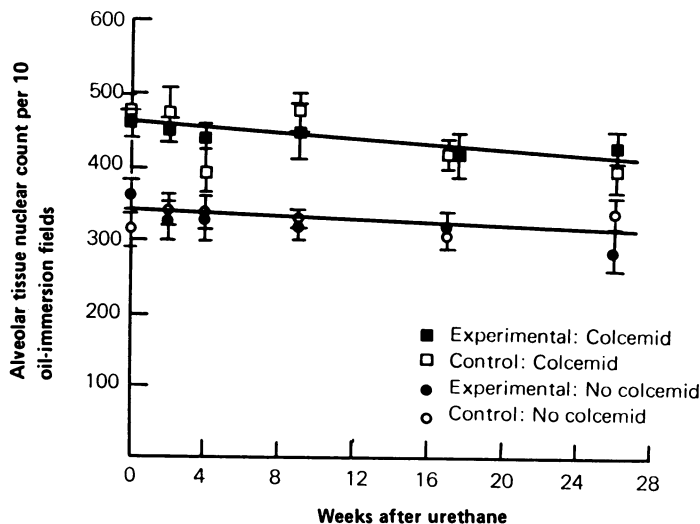


FIG. 8.—Alveolar tissue nuclear count in relation to survival.

TABLE.—*Kinetic Parameters of Adenomata in Relation to Survival*

Time PU (weeks)	*R _M % per h	I _M %	t _M h	R _S % per h	I _S %	t _S h
4	—	0.88	—	3.0	2.8	0.93
9	0.185	0.15	1.35	1.2	1.8	1.5
17	0.075	0.24	0.32	0.8	1.4	1.75
26	0.055	0.09	1.64	0.3	1.2	4.0

* Calculated on the assumption that Colcemid does not arrest cells already in metaphase at the time of its administration.

and R_S demonstrate that k_B is falling with time, which could in turn be caused by a decrease in growth fraction (I_p) and/or an increase in cell cycle time (t_C). The Table shows that t_S increased markedly with time, suggesting that t_C could also be increasing. However, the value of 1.4 h for t_S is short in comparison with that of 5–10 h given by Cleaver (1967) for mouse tissues. From the Table it can be seen that R_S differs from R_M by factors of between 5 and 11. It is possible that diurnal effects are contributing towards this discrepancy. However, Bertalanffy *et al.* (1965) could find no evidence from their work, or from the literature, for any diurnal variation in proliferative activity in experimental animal tumours. It is also pertinent that Bertalanffy (1964) found no diurnal variation in mitotic rate in normal rodent alveolar wall cells. Another possibility is that Colcemid is somehow interfering with labelling. This is unlikely since Puck and Steffen (1963) showed that Colcemid had no effect on the G₁, G₂ and S phases of HeLa cells *in vitro*. The discrepancy between R_S and R_M is the subject of further investigation.

Surface adenomata could represent a faster growing sub-population appearing early on the lung surface, and they might not be comparable with sectioned adenomata, from which metaphase and labelling indices were obtained. However, this objection is unlikely for several reasons. Stewart (1959) noted that most adenomata are subpleural in origin and subsequent growth makes them readily ap-

parent on the lung surface. Our use of a dissecting microscope and strong reflected light permitted recognition of adenomata when they were no larger than 150–200 μm in diameter and situated at some depth in the lung parenchyma. Moreover, Shimkin and Polissar (1955) estimated from serial lung sections that, by 105 days PU, 50% of the adenomata in mouse lungs were visible on the lung surface to the naked eye alone. The growth pattern exhibited by our surface adenomata correlates well with that observed by Shimkin and Polissar (1955) for serially sectioned adenomata. All these considerations suggest that the surface adenomata do in fact accurately reflect the growth of the whole adenoma population of the lung.

Alveolar wall tissue

An increase in proliferative rate occurs within the first month following urethane treatment. This response reaches a peak at 2 weeks but disappears by about 2 months PU. During this response I_{DL}, I_S and I_M are all elevated but no corresponding increase in I_{M(a)} could be detected. Shimkin and Polissar (1955) noted a progressive increase in the number of alveolar wall cells 1–28 days after urethane injection; a diffuse hyperplasia and small subpleural clusters of enlarged cells were found after 21 days but the latter gradually disappeared as adenomata became apparent. Foley *et al.* (1963) demonstrated an increased DNA synthetic index in alveolar tissue 2–7 days following urethane injection; this proliferative response and the numbers of adenomata induced were decreased by x-irradiation of the lungs before urethane treatment. These observations raise the question as to whether the urethane induced proliferative response is a necessary precursor of adenomata as the results of Foley *et al.* (1963) suggest. The appropriate cell population is not, however, being studied since adenomata are derived from type II alveolar epi-

thelial cells (Svoboda, 1962), whereas alveolar walls also include type I cells, interstitial cells and endothelium. The majority of cells taking part in the urethane induced proliferative response in alveolar walls appear to be of the non-vacuolated type, possibly replacing cells killed by the toxic effects of urethane (Kaufmann, 1969). Shimkin *et al.* (1969) believed that the proliferative response in alveolar walls was a side-effect unrelated to urethane carcinogenesis, since urethane doses of 0.25 mg and 1.0 mg/g body weight induced adenomata but only the latter dose increased the pulse labelling index in alveolar walls. However, Kauffman (1974) has recently shown that there is a doubling in the size of the type I, type II and alveolar macrophage populations from 2 to 6 weeks after the beginning of chronic exposure to urethane. Thus, growth of type II cells is evidently a component of the hyperplasia accompanying urethane exposure.

I_S was often elevated above I_{DL} in alveolar walls (Fig. 7), making it impossible to calculate R_S and t_S , but in the adenomata I_{DL} was consistently above I_S (Fig. 5). It is therefore unlikely that Colcemid is interfering with DNA synthesis and hence with labelling. When the nuclear count of alveolar tissue is plotted against time (Fig. 8) Colcemid is seen to cause an increase in cellularity. There was no suggestion that Colcemid caused collapse of lung and so augmented the cell population per field. On the other hand, Colcemid might promote immigration of circulating cells from the blood into the alveolar wall. Dixon and Malden (1908) reported that colchicine produced a leucocytosis in rabbits and dogs, while vincristine caused a rapid leucocytosis and a decrease in the cellularity of the bone marrow in rats (Frei *et al.*, 1964). Stathmokinetic agents could thus cause a rapid release of leucocytes from the bone marrow into the blood and some leucocytes might be sequestered in the alveolar tissue. When $^3\text{H-TdR}$ is given at the same time as Colcemid,

some of these leucocytes might become labelled if they were immature and still synthesizing DNA. The increase in the single labelling index of alveolar wall tissue (Fig. 7) may be explained in this way.

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