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The Measurement of Cell Production Rates in the Crypts of Lieberkuhn

An Experimental and Clinical Study

Nicholas Wright, Alexander Watson, Adrian Morley, David Appleton, Janet Marks, and Adrian Douglas

Departments of Pathology, Medical Statistics, Dermatology and Medicine, University of Newcastle upon Tyne

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Summary. A method is described of assessing the proliferative state in the small bowel mucosa employing the metaphase-arresting (stathmokinetic) properties of vincristine, applicable to both animals and man. In the rat a value for the migration rate of 1.78 cell positions per hour was obtained by the stathmokinetic method, in agreement with a further measurement of the migration rate made using tritiated thymidine. Values for the transit time through the crypt (34 hours) and for the cell production rate (39 cells per crypt per hour), are in good agreement with previously published values.

A control patient and a patient with the flat mucosa of gluten-sensitive enteropathy were also studied by the vincristine technique. The migration and cell production rates were markedly increased in the flat mucosae. A hyperproductive proliferative state is therefore confirmed in the flat mucosa of untreated gluten-sensitive enteropathy.

The results show the vincristine technique to be eminently applicable to problems involving intestinal cell kinetics.

Introduction

In recent years the detailed work of Lipkin (1965) has allowed estimation of the time parameters of the cell cycle in the human small bowel by the use of techniques involving tritiated thymidine in individuals with disseminated malignancies; unfortunately such techniques are too hazardous for routine use. In view of the debate concerning the mucosal proliferative state in the gluten-sensitive enteropathies (Creamer, 1962; Booth, 1970), and because of the current interest in the influence of irradiation and cycle specific chemotherapeutic agents on crypt cell kinetics (Skipper and Schabel, 1971; Lamerton, 1972) it has become necessary to evolve a simple technique by which mucosal dynamics can be studied in routine peroral biopsies.

The most satisfactory single proliferative parameter which has emerged from recent work on small bowel mucosal kinetics is the cell production rate per crypt (Clarke, 1971). This measurement is the resultant of many interrelated kinetic parameters; for example changes in growth fraction or in cell cycle time are reflected in the cell production rate. In the rat, Clarke has measured cell production rates by a stathmokinetic technique involving colcemid. We have evolved an alternative method of measuring cell production rate; a method which also yields information on several other important proliferative parameters. In this paper we substantiate the validity of the method experimentally and demonstrate its clinical applicability.

Materials and Methods

I. Experimental Material

Male albino Wistar rats aged 3 months and weighing 150–200 g were used throughout. All injection procedures were carried out at 09.00 h. Twelve animals were given 0.5 μCi of tritiated thymidine (^{3}HTdr) per g body weight by intraperitoneal injection. The ^{3}HTdr was obtained from the Radiochemical Centre, Amersham, England, and was of specific activity 5.0 Ci/mM. The concentration used was 100 μCi/ml. Samples of small bowel were taken from the region immediately distal to the ligament of Treitz, and would thus be described as upper jejunum. The tissue was fixed in Carnoy's fluid for 6 h, paraffin embedded, and serial transverse sections were cut at a thickness of 3 μm. Autoradiographs were prepared as previously described (Wright, Morley and Appleton, 1972a), using Kodak AR 10 stripping plates and an exposure time of 28 days.

In each animal 100 crypts were selected. Only complete axial crypt sections were chosen i.e. those in which the base, middle and mouth of the crypt were all present in the plane of section. Care was taken to ensure that each crypt was analysed only once. The counting techniques of Cairnie, Lamerton and Steel (1965a) were used throughout. The identity of the sections was unknown to the observer. In each crypt the left hand column of cells was numbered, counting from the bottom upwards to the crypt-villus junction, and the cell positions of labelled nuclei were recorded. During crypt analysis the number of cells present in a column was also recorded; this is the *crypt column*, and a further morphometric parameter was measured, the *column count* (Wright, Watson, Morley, Appleton and Marks, 1973). This was carried out in three untreated animals by cutting sections tangential to the bowel lumen so that crypts were cut in transverse section. In each animal the column count was estimated from counts on 100 approximately circular crypt cross sections.

II. Clinical Material

Patient A was a male aged 61, with a clinical history of rosacea. On day 1 a modified Crosby capsule was passed, and a mucosal biopsy of the duodeno-jejunal region was obtained in the usual way at 13.30 h. On day 2 the capsule was again passed and at 10.45 h was adjudged radiologically to be in the same position as on the previous day. At 11.00 h vincristine sulphate was rapidly infused intravenously in a dose of 0.045 mg/kg body weight. At 13.30 h precisely the capsule was fired and a mucosal biopsy obtained. Care was taken to ensure that no appreciable movement of the capsule occurred during the stathmokinetic period following vincristine injection.

Patient B was a male aged 37, with a ten year history of dermatitis herpetiformis. Two mucosal biopsies were obtained following the same protocol as that outlined above, again ensuring that the duration of the stathmokinetic period was exactly 2.5 h.

The biopsies were spread flat under the stereomicroscope and fixed for 24 h in neutral buffered formol saline and for a further 24 h in $HgCl_2$-formol solution. Serial paraffin sections were cut at a thickness of 3 μm and stained with Harris's haematoxylin. In patients A and B quantitative analysis was carried out in the manner already described and the column count was obtained as before from sections cut tangentially to the bowel lumen.

Both patients received full information concerning the investigative procedure and informed consent was obtained in each case; careful follow up has revealed no evidence of harmful effects due to vincristine.

In any sample of small intestinal crypts considerable variation is found in the height of the crypt columns; in calculating the mitotic index (or labelling index) as a function of cell position such variation should be taken into account. Cairnie and Bentley (1967) found differences in the distribution of labelled cells in short and long crypts of normal animals depending on whether the positions of cells were taken relative to the top or bottom of the crypt; clearly the same argument applies to the distribution of mitoses. For this reason we have analysed the data by a

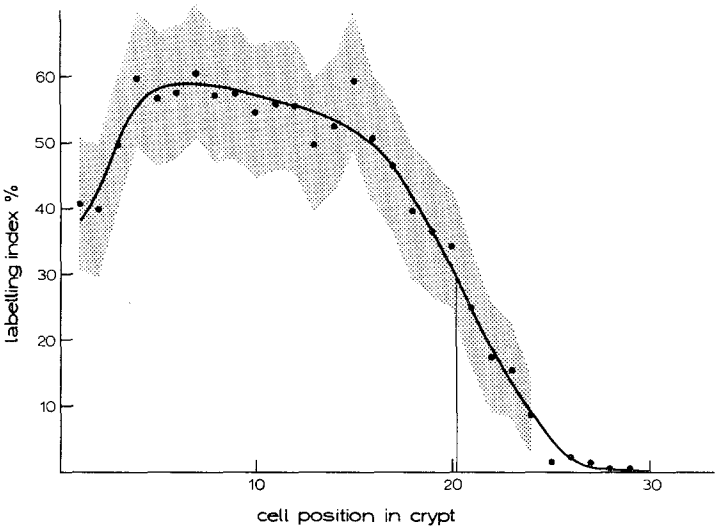

Fig. 1. Labelling index distribution curve for the 3 month old male Wistar rat. The shaded area indicates 95% confidence limits for the points, and the perpendicular shows the cell positions of the 50% peak value

modified version of the method designed by Cairnie and Bentley (1967), subjecting it to an ALGOL program run on an IBM 360/67 computer. Details of this procedure, and the method employed for the construction of *labelling and mitoitc index distribution curves*, are given elsewhere (Wright *et al.*, 1972a).

A nucleus was regarded as being labelled if 5 or more grains were localised over it (Wright, 1971). The morphological criteria used for the recognition of the various mitotic stages were similar to those of Clarke (1970).

Tannock (1967) has indicated that an important geometrical factor is involved in the measurement of the crypt mitotic index from tissue sections. Nuclei in mitosis are out of line with the interphase nuclei of the remainder of the crypt column, and are found to be nearer the crypt axis. This spatial arrangement leads to an overestimate of the mitotic index. Tannock therefore proposed a constant, a/b (where a is the radial position of mitoses in the crypt, and b is the radius of the crypt lumen), by which the mitotic index should be multiplied in order to correct for this error. Sections cut tangentially to the bowel were therefore examined. In the rat a value of the ratio a/b was obtained by measurement of 210 approximately circular crypt sections containing metaphases, using a calibrated eyepiece graticule. In patient A we measured 102, and in patient B 106 crypt cross sections.

Results and Interpretation

I. ^{3}*HTdr Studies in Rats*

In Fig. 1 is shown the labelling index (I_L) plotted as a function of the cell position in the crypt for two animals killed 1 h after ^{3}HTdr administration. Initially, at the base of the crypt lower indices are found, for example 40% at

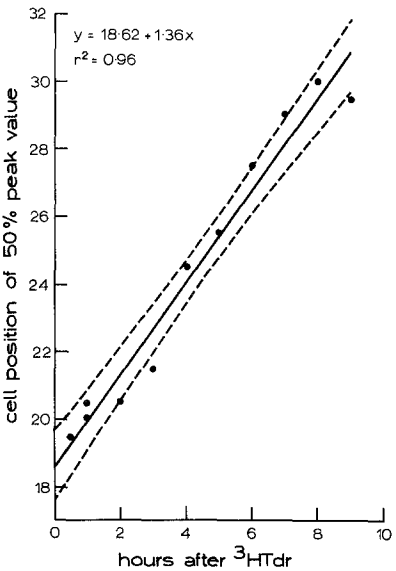

Fig. 2. Movement of the 50% peak value with time after injection of ^{3}HTdr. The solid line was fitted by linear regression analysis, and the dotted lines indicate 95% confidence limits for the line. The slope is then a measure of the migration rate

cell position 1. Cells in this portion of the crypt have previously been shown to have lower proliferative indices (Cairnie *et al.*, 1965a) and Wright, Morley and Appleton (1972b) have demonstrated a prolonged cell cycle time in these cells. They may well constitute a 'stem-cell compartment' for the contiguous proliferative compartment (Lamerton, 1972). A rapid increase in I_L then occurs to reach a maximum value of 60% at cell position 7. A steady decrease then follows, and very low indices are reached by cell position 25.

The growth fraction, or proliferating pool (I_p) calculated for the whole crypt represents the fraction of the crypt population that is in the proliferative compartment. The fraction of the crypt occupied exclusively by the *proliferative compartment* is the region over which labelled or mitotic cells are observed, and this region was considered to end approximately at the cell position where the I_L fell to 50% of the maximum or peak value (Cleaver, 1967). This was found to be at cell position 20. The I_p is equal to the ratio of this to the number of cells in the crypt column, i.e. 20/33, or 0.61. That is to say 61% of the crypt is occupied by the *proliferative compartment*, and the remainder by the maturation *compartment*, composed of differentiating cells. This compares well with an I_p value of 0.62 calculated from the duration of cell cycle phases and the labelling index (Wright, Aldewachi, Appleton and Watson, in preparation).

By taking the cell position of the 50% peak value as a reference point an estimate can be obtained of the cell migration rate. I_L distribution curves were constructed for each animal killed up to 9 h after ^{3}HTdr administration. After 9 h labelled cells began to appear on the villi. Fig. 2 shows the movement of

the 50% peak value with time after ^{3}HTdr administration. The two dotted lines are 95% confidence limits for the line. The slope of the line gives the cell migration rate expressed in cell positions traversed per h, and was equal to 1.36 ± 0.19 (95% confidence limits).

In a previous study (Wright *et al.*, 1972), we described a technique employing vincristine in which cumulative birth rate curves were constructed for the rat jejunal crypt from accurate values for the mitotic duration at each cell position. This method gives a cell velocity of 1.78 cell positions per h, in reasonable agreement with the value given by the ^{3}HTdr technique described above.

Table 1. Compartmental sizes and proliferative populations in the rat jejunal mucosa

Crypt column (cells)	Column count (cells)	Crypt population (cells)	Growth fraction	Number of proliferating cells
32.9 ± 0.2	22.3 ± 0.3[a]	734	0.61	448

[a] Standard error.

Table 1 shows the morphometric parameters obtained in the rat. The product of the crypt column and the column count gives an estimate of the total crypt population (Wimber and Lamerton, 1963; Cairnie and Bentley, 1967), and as calculated from the present data the crypt population is 734 cells. The growth fraction has been derived from the I_L distribution curve, and the product of the I_p and the total crypt population should give an estimate of the total number of proliferating cells per crypt. This is 448; the remaining 286 cells are differentiating cells occupying the maturation compartment.

A further kinetic parameter which can be calculated from these cummulative birth rate curves is the transit time (T) through each compartment. The transit time past any particular cell position is equal to the reciprocal of the birth rate at that cell position. If the compartment sizes are known, then calculation of their respective transit times can be found by summation. These are shown in Table 2. The crypt transit time (exclusive of 'stem cells') is 34 h. Assuming the first three cell positions to be 'stem cells' for the proliferative compartment (see discussion) the transit time for the proliferative compartment is 26 h, and for the maturation compartment 8 h.

Table 2. Flux parameters in the rat jejunal mucosa

Migration rate (cell positions per h)	Crypt cell production rate (cells/crypt/h)	$T_{\text{prolif.}}$ (h)	$T_{\text{mat.}}$ (h)	$T_{\text{prolif.+mat.}}$ (h)
1.78[a] 1.36[b]	39	26.1	7.9	34.0

[a] From cumulative birth rate curve.
[b] From movement of 50% peaks after labelling with ^{3}*HTdr*.

As already explained, the limiting value of the cumulative birth rate curve is equal to the efflux for a single crypt column, and in effect represents the rate of cell production for that column. If this value is multiplied by the total number of columns in the crypt (the column count), then a value for total cell production rate per crypt is obtained. This is equal to 39 cells per crypt per hour (Table 2), and is in good agreement with the value of 35 cells per crypt per hour obtained from microdissected crypts by Clarke (1971), also for the Wistar rat. Wright *et al.* (in preparation) also studying microdissected and squashed crypts, calculated values for crypt cell production rate of about 34 cells per crypt per h from measurements of the durations of the phases of the cell cycle and of the growth fraction. The present value at 39 may be slightly higher because sections have been employed (Clarke, 1968).

II. Vincristine Studies in Humans

The biopsies from patient A showed finger-shaped and narrow leaf shaped villi only, and histological examination showed no notable abnormality. Patient B showed a flat mucosa with a cobblestoned appearance in both biopsies, and the histological appearances were those of total villous atrophy. In each case the post-vincristine sample showed no evidence of anaphase escape, i.e. no anaphases were detected, and metaphase blockade was assumed to be complete.

In Table 3 are shown the morphometric parameters for the two cases, and it is evident that in addition to the well known lengthening of the crypts in flat mucosae there is also a considerable increase in the girth of the crypts. In flat mucosae there is clearly a *three-dimensional increase* in crypt size (Wright *et al.*, 1973), and this is reflected in the value obtained for the crypt population, an almost three-fold increase.

The I_m distribution curve constructed from the pre-vincristine sample from patient A is shown in Fig. 3a. The I_m rises from very low values of below 0.5% in the basal cell positions to a peak of nearly 4.0% at cell position 9; values above 3.0% are found thereafter until cell position 23, where there is a rapid fall to low values. As mentioned above, Cleaver (1967) considers that the proliferative compartment is that region where labelled *or mitotic* cells are observed, and here we are obliged to measure I_p from I_m distribution curves. As would be expected on the basis of the slow cut-off model (Cairnie, Lamerton and Steele, 1965b), this method gives higher values than those obtained from I_L distribution curve; however, since the I_m distribution curve is used throughout the human studies, direct comparison can be made. The 50% peak value is at cell position 26, and the I_p is therefore 0.81. The I_m distribution curve for the post-vincristine sample is shown in Fig. 3b, and although considerable increases are evident in individual I_m values at each cell position, it can be seen that there is no apparent upward displacement of the 50% peak values.

The I_m distribution curve for the pre-vincristine sample from patient B is shown in Fig. 3c. Again low values are evident in the lower cell positions, but a rapid rise to a higher peak value of over 10% between cell positions 11 and 22 then occurs, and is followed by a steady fall to low I_m values again towards the top of the crypt. The 50% peak value is at cell position 40 and this gives an I_p

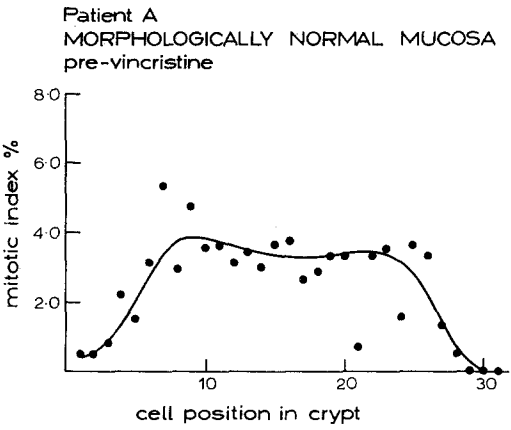

Fig. 3a

Fig. 3a—d. Mitotic index distribution curves, 3(a) for the previncristine, and 3(b) for the post-vincristine biopsies from patient A. 3(c) and 3(d) show the mitotic index distribution curves for the pre and postvincristine samples respectively for patient B. Note the increase in I_m at each position in the postvincristine samples, but without movement of the 50% peak value. The shaded areas indicate 95% confidence limits for the points

Table 3. Morphometric parameters in human small bowel mucosa; compartment sizes and proliferating populations in human small bowel mucosa

	Crypt column (cells)	Column count (cells)	Crypt population (cells)	Growth fraction	Number of proliferating cells/crypt
Patient A (normal villous mucosa)	31.9 ± 0.2	22.3 ± 0.6	711	0.81	576
Patient B (flat avillous mucosa)	67.0 ± 0.5	30.4 ± 0.5	2036	0.60	1222

	Proliferative compartment size (cells)[a]	Maturation compartment size (cells)[a]	Mitotic index (%)
Patient A	26 (81%)	7 (19%)	2.36
Patient B	40 (60%)	37 (40%)	5.09

[a] Measured from the previncristine I_m distribution curves.

value of 0.60. The I_m distribution curve obtained from the post-vincristine sample is shown in Fig. 3d; as expected there are considerable increases in values of I_m at individual cell positions; but again, there has been no apparent upward movement of the 50% peak values during the period of metaphase arrest. This demonstrates that vincristine effectively inhibits cell division and thus cell migration.

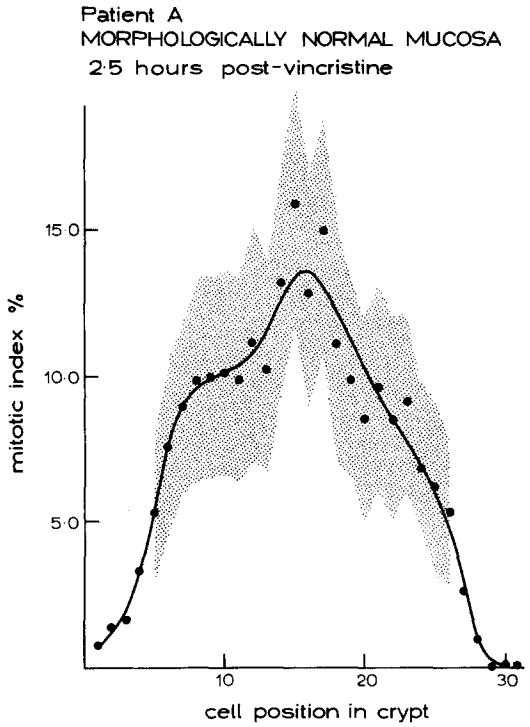
Patient A
MORPHOLOGICALLY NORMAL MUCOSA
2·5 hours post-vincristine
15·0
10·0
5·0
mitotic index %
10
20
30
cell position in crypt

Fig. 3b

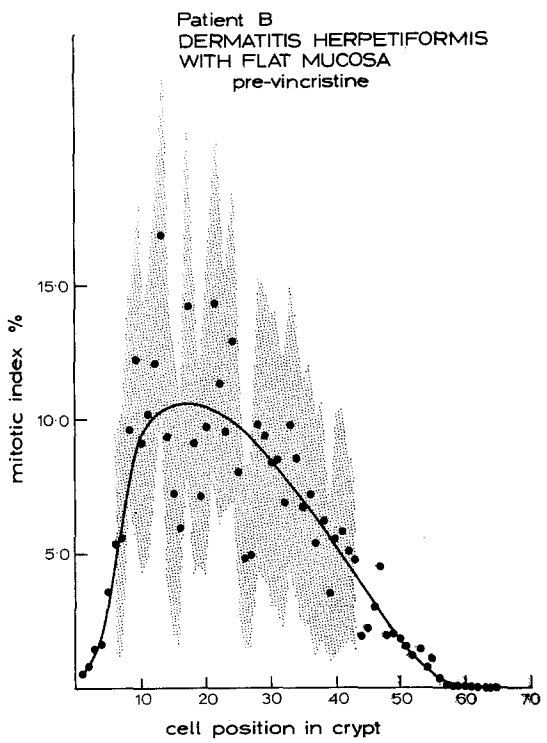
Patient B
DERMATITIS HERPETIFORMIS
WITH FLAT MUCOSA
pre-vincristine
15·0
10·0
5·0
mitotic index %
10
20
30
40
50
60
70
cell position in crypt

Fig. 3c

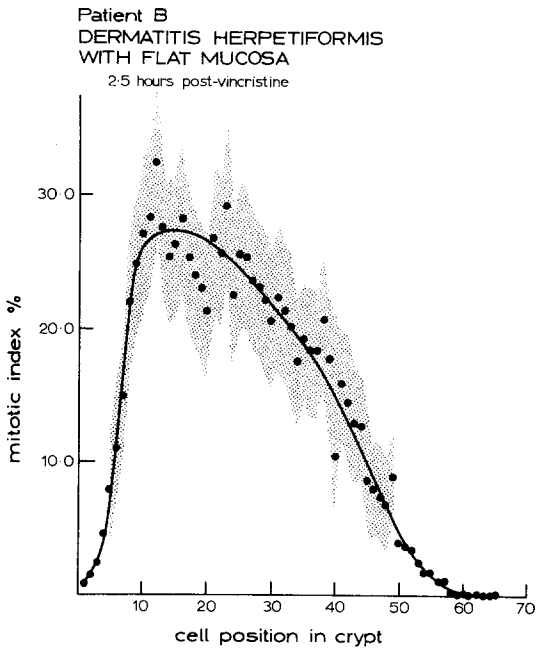

Fig. 3d

Values for the proliferative and maturation compartment sizes obtained from the pre-vincristine I_m distribution curves are given in Table 3. Despite a small decrease in I_p in patient B, because of the pronounced three dimensional increase in crypt size, there was an approximately two-fold increase in the number of proliferating cells per crypt. That is to say, that although there is a *relative* reduction in proliferative compartment size in the flat mucosa of patient B, an absolute increase in proliferative compartment size (in terms of cell number) has occurred because of the length (and girth) increase in the crypts of the flat mucosa. I_m values for the whole crypt column (obtained from pre-vincristine samples) are also given in Table 3. I_m was significantly higher ($p < 0.001$) in patient B, and this difference is of course accentuated when the I_m is calculated as a percentage of the proliferating population only. The values obtained for Tannock's constant were 0.71 for patient A and 0.72 for patient B.

The duration of mitosis, t_m, was calculated at each position (Wright *et al.*, 1972b) and cumulative birth rate curves constructed; these are shown for patient A in Fig. 4a, and for patient B in Fig. 4b. The migration rate was 0.46 cell positions per h in patient A, and 1,65 in patient B (Table 4). Estimates of cell production rate show a four to five-fold increase in patient B. The transit times through the various compartments are given in Table 4, and it can be seen that despite the increased length of the crypt column in the flat mucosa of patient B, the transit time through the proliferative compartment is shorter. This is of course a reflection of the increased migration rate in patient B. The stem cell compartment was defined as those cells at the base of the crypt with transit times exceeding 20 h.

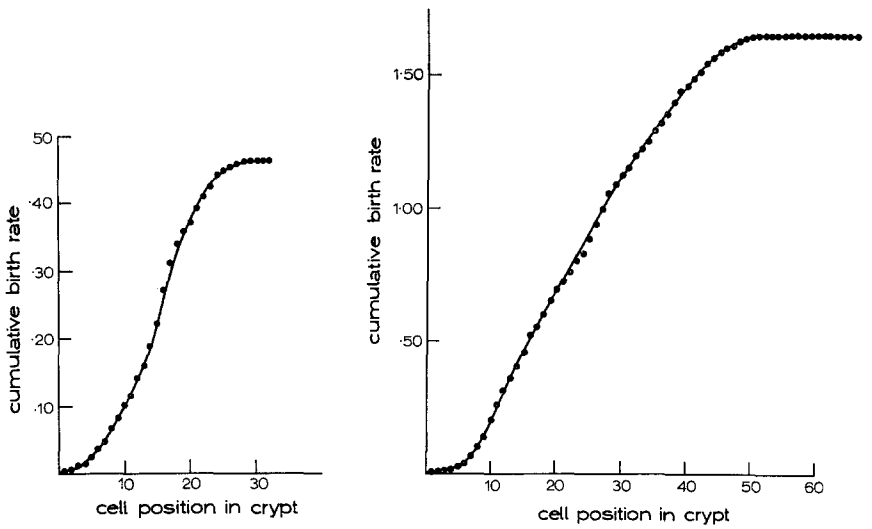

Fig. 4. Cumulative birth rate curves for patient A (4a), and patient B (4b). In each case the t_m values obtained from the vincristine study have been used in the calculation of the birth rate at each cell position

Table 4. Flux parameters in the human small bowel mucosa

	Migration rate (cell positions/h)	Crypt cell production rate (cells/crypt/h)	$T_{\text{prolif.}}$ (h)	$T_{\text{mat.}}$ (h)	$T_{\text{prolif.+mat.}}$ (h)
Patient A (villous mucosa)	0.46	10.2	116	15	132
Patient B (flat avillous mucosa)	1.65	50.2	60	15	75

This involved the lower 6 cell positions in each case. A similar principle was employed by Wright *et al.* (1972b) in their definition of rat stem cells.

Implicit in the above calculations are two assumptions concerning the mode of action of vincristine. Firstly that vincristine exerts its stathmokinetic (or metaphase arresting) action immediately upon injection, as is apparent in the rat studies; if vincristine does not block cells which had already embarked upon mitosis there would be a lag equal to the duration of mitosis before metaphase accumulation began, with consequent underestimation of the rate of entry into mitosis. The second assumption is that linear accumulation of metaphases occured. Both these assumptions have been confirmed using the multiple biopsy technique after vincristine injection (Wright, Watson, Morley, Appleton, Marks and Douglas, 1973).

Discussion

From the animal studies here described it is clear that the vincristine technique gives values for the crypt cell migration rate which compare well with ^{3}HTdr methods. Further, the estimate obtained for cell production rate (39 cells per crypt per h) is in agreement with that of Clarke (1971), and of Wright *et al.* (1974), who both used the crypt squash technique. One assumption implicit in the present method is that the crypt approximates to a cylinder, but the conformity of the cell production rates would appear to vindicate this assumption in this context.

An important point in the construction of the cumulative birth rate curves is the choice of t_m. Wright *et al.* (1972b) have explored the variation in migration rate when constant values for t_m are assumed to obtain at all cell positions; they concluded that accurate values for migration rate could only result when t_m is measured at each cell position and this variable t_m used in the calculation. The procedure is, of course more laborious than the crypt squash method, and can be criticised concerning the assumption made above; in its favour the method also gives information concerning the rate of migration of cells at all points within the crypt, a comparative estimate of the proliferating population, and estimates of transit time through the various compartments. Also, in our hands, reasonable values for crypt cell production rate have been obtained.

It should be noted that the value obtained for the migration rate using the movement of the 50% I_L peaks is slightly higher than that of Cairnie *et al.* (1965a); it may also be more accurate, as more crypts were enumerated in the present study, and the scatter of points about the line is reduced.

The problem of delineating the stem cell compartment at the base of the crypt is one which has been considered by several investigators (Cleaver, 1967; Lamerton, 1972) who have defined it on purely arbitrary grounds. Wright *et al.* (1972b) have studied the kinetics of these cells at the base of the crypt and showed that the bottom three position have a longer cell cycle time than that of cells in the proliferative compartment. It is possible that these longer cycling cells act as stem cells for the small bowel in the same way as haemopoietic stem cells (also of long cycle duration).

As in the animal studies, t_m calculated at each cell position was used in the construction of cumulative birth rate curves for the most accurate results in the human work; it is evident that there is a four-fold increase in migration rate, and, again assuming a tubular crypt structure in both control and flat mucosae, a four to five-fold increase in cell production rate in the flat mucosa of patient B. Although this assumption appears reasonable from observation of serial transverse crypt sections in each case, some accurate stereological model building would be required for complete justification.

The increased migration rate confirms the observations of Trier and Browning (1970) who, using an *in vitro* culture technique, involving labelling with tritiated thymidine, were able to demonstrate faster migration rates in biopsies from patients with the flat mucosa of gluten-sensitive enteropathy. On the other hand, there is nothing to support the conclusions of Creamer (1962) who suggested that crypt cells in flat mucosae moved upwards at a slower rate than normal crypt cells. The present results, together with those of a previous study (Wright *et al.*, 1973) indicate a hyperproductive proliferative state in flat mucosae.

It should be noted that the kinetic arguments presented all incorporate a tacit assumption of steady state conditions. The equations are readily altered to fit exponential conditions; steady state is a reasonable assumption in the normal mucosae (Schultze, Staack, Schmeer and Maurer, 1972), and while we have no prior indications of the growth conditions to be expected in flat mucosae, there is no reason to suppose that steady state conditions do not obtain.

As already mentioned, the use of mitotic index distribution curves to estimate the growth fraction would be expected to give higher values than those obtained from labelling index distribution curves (Wright *et al.*, 1973) and this is borne out by the observations of Cairnie *et al.* (1965b) on the basis of the slow cut-off model. However, this usage can be expected to give a maximum value for the growth fraction and to demonstrate any major differences in proliferating population between two contrasted groups. An earlier experimental study (Wright *et al.*, 1972b) showed that the differences between I_p measured from labelling index and from mitotic index distribution curves is not large; in the human studies the sizes of the proliferative compartments should therefore be regarded as maximum values.

In the human studies the delineation of the 'stem cell compartment' is difficult and needs some assumptions. The stem cell compartments in patients A and B have arbitrarily been defined on the basis of transit time; all cell positions with transit times exceeding 20 h being incorporated in the 'stem cell compartment'. This involves the lower 6 cell positions in both cases A and B. Further work is being undertaken to characterise this important portion of the crypt more accurately, considering Paneth cells and using techniques established experimentally in a previous study (Wright *et al.*, 1972b).

Vincristine appears to be an excellent agent for studying small intestinal cell kinetics in animals and man. In each study there was no evidence that cells were passing the metaphase blockade, and because of the short arrest period employed, there was no apparent loss of metaphases due to degeneration (Aherne and Camplejohn, 1972). No evidence of deleterious side effects has been found in seven of our patients given the drug, and Camplejohn, Bone and Aherne (1973) have used the technique in 25 patients without mishap. Since *in vivo* techniques involving tritiated thymidine are potentially too dangerous for routine use, and crude mitotic indices are far from satisfactory, we suggest that the technique described is most suitable for the detailed investigation of small bowel proliferative status.

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Ethical Considerations. All patients received a full explanation of the procedure before informed consent was obtained. The project was presented to the Ethical Committee of the Newcastle University Hospital Group, and was approved under the following terms of reference.

1971. Dr. J. Marks. The use of vincristine with two Crosby capsule biopsies.

1972. Dr. A. Douglas. The use of vincristine in conjuction with the Quinton multiple biopsy machine.

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Dr. Nicholas Wright
Department of Pathology
Newcastle University Hospitals
The Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne, NE1 4LP, England