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A geometric model for estimating villous surface area in rat small bowel is justified by unbiased estimates obtained using vertical sections

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INTRODUCTION

In an earlier study (Mayhew, 1984), a geometric model was proposed for estimating the extent to which villi (v) amplify the surface area (S) of primary mucosa (pm) in different regions along the rat small bowel. The model was exploited to investigate regional differences in control and fasted animals and to examine the effects of fasting (Mayhew & Middleton, 1985; Ross & Mayhew, 1985; Mayhew, Middleton & Ross, 1988). In essence, the model assumes that the primary mucosa comprises an anisotropic (i.e. a convex cylindrical) tube on the inner aspect of which villi form a surface which is isotropic overall.

With this model, the surface amplification, $S(v)/S(pm)$, could be estimated from intersection ratios, $I(v)/I(pm)$, taken from transverse and/or longitudinal sections through small bowel. If the model holds, a correction factor of $4/\pi$ is appropriate for intersection ratios taken from transverse sections and one of $8/\pi^2$ for ratios taken from longitudinal sections. Cutting only transverse sections has two main advantages: it provides a useful visual impression of bowel architecture and it helps to reduce sampling variation.

The model proposed can be tested against unbiased estimates obtained by design-based sampling. Several sampling strategies which are free from assumptions about surface orientation properties would suffice. Currently available are: (*a*) directionally independent isotropic uniform random (IUR) sectioning, (*b*) IUR-orientated sectioning using orthogonal triplet probes and (*c*) vertical sectioning (see Mattfeldt, Mobius & Mall, 1985; Baddeley, Gundersen & Cruz-Orive, 1986).

In this study, vertical sectioning was elected as the most practicable and economical candidate for dealing with small bowel since suitably sampled longitudinal sections employed in an earlier study (Mayhew, 1984) could also be used as vertical sections. Therefore, estimates of $S(v)/S(pm)$ generated by analysing vertical sections were compared with those obtained from transverse and longitudinal sections after applying corrections.

MATERIALS AND METHODS

Tissue preparation and sampling

Full details about the animals employed and about tissue preparation steps are given elsewhere (Mayhew, 1984). Briefly, six adult rats of group mean body weight 300 g were used. Under ether anaesthesia, all were killed by intracardiac perfusion with isotonic saline prewash followed by Millonig's phosphate-buffered glutar-

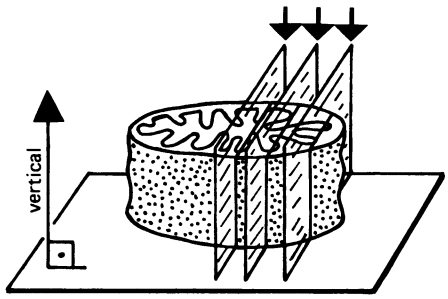

Fig. 1. Sketch illustrating a segment of small bowel cut by a set of three systematic random vertical sections. The base represents a 'horizontal' plane which is parallel to the top and bottom transverse sections across the bowel. Vertical sections therefore run parallel to the long axis of the bowel (i.e. they correspond to longitudinal sections). The vertical sections must be randomly located and randomly (isotropically) rotated on the horizontal plane.

aldehyde (pH 7·3) at room temperature. After fixation, each bowel was removed and 2 cm lengths taken from proximal, middle and distal regions. This arbitrary sampling scheme suffices for making internal comparisons within a study but would be inappropriate for estimating quantities for the small bowel as a whole (Mayhew & Middleton, 1985; Mayhew *et al.* 1988)

Each 2 cm length was sliced transversely and the pieces transferred to buffered osmium tetroxide prior to resin-embedding in flat-bottomed containers. Later, pieces were glued to resin blanks so that each could be cut transversely to the bowel long axis.

One block per segment was selected by lottery and a complete, arbitrarily-situated transverse section was cut. Subsequently, each block was re-orientated on a new blank and cut longitudinally (i.e. parallel to the central axial direction of the bowel). In the present context, the terms 'longitudinal' and 'vertical' can be regarded as being synonymous, the former (latter) term being used to denote sections analysed by the model (design)-based approach. Each set of sections was selected as a systematic random sample of the complete series passing from the bowel periphery to the centre (see Fig. 1 and Mayhew, 1984). Provided the direction of approach is randomised (i.e. bowel segments are rotated about their central axes), this scheme meets the requirement (Baddeley *et al.* 1986) that vertical sections must have random locations on the horizontal (here represented by the transverse) plane and random orientation about the vertical direction. Between two and four vertical/longitudinal sections per bowel segment were sampled. All sections were roughly 0·5 μm thick and stained with toluidine blue.

Each section was projected on to a sheet of drawing paper at a final enlargement of $\times 50$. To ensure unambiguous recognition of feature contours, boundary traces of villous and of primary mucosal surfaces were drawn in pencil. Drawings were made for all section sets (transverse and longitudinal/vertical) sampled from each bowel segment. Test lattices were superimposed on each drawing in turn to obtain test intersection totals with traces of villi $I(v)$ and with traces of primary mucosa $I(pm)$. In the case of model-based estimators, test lattices consisted of equidistant parallel straight lines superimposed initially so as to be independent in position and orientation. The first orientation was taken to represent a 0° axis and lattices were applied repeatedly at orientations up to 150° using equiangular increments of 30° (Mayhew, 1984). For vertical section estimators, test lattices consisting of cycloids (Baddeley *et al.* 1986, staggered lattice in their Fig. 7*b*) were superimposed so as to be

independent in position although orientated so that the vertical axis of the test lattice coincided with the bowel long axis (Baddeley *et al.* 1986). Five superimpositions of the lattice were made on each tracing.

Estimating surface amplifications due to villi

The following relationships for estimating $S(v)/S(pm)$ were invoked:

(1) *Model-based estimations*

(*a*) *Transverse sections.* Intersections $I(v)$ and $I(pm)$, counted using straight test lines were summed over all test lattice orientations on each drawing and the relationship

$$S(v)/S(pm) = (4/\pi).I(v)/I(pm)$$

employed to estimate surface amplification (Mayhew, 1984).

(*b*) *Longitudinal sections.* Intersection counts for systematic random longitudinal sections were summed over all orientations of straight test lines and over all sections. Then,

$$S(v)/S(pm) = (8/\pi^2).I(v)/I(pm)$$

was used to estimate surface amplification (Mayhew, 1984).

(2) *Design-based estimation*

Intersections with cycloid arcs counted on longitudinal (= vertical) sections were summed over all five superimpositions on all sections and

$$S(v)/S(pm) = I(v)/I(pm)$$

was taken to be the unbiased estimate of surface amplification. This relationship follows from the fact that any surface $S(c)$ in a reference volume $V(r)$ can be estimated from vertical sections using the stereological principle

$$S(c)/V(r) = 2.I(c)/L(r),$$

where $L(r)$ denotes the total length of cycloid arcs falling on the reference space $V(r)$ and over which intersections $I(c)$ occur (consult Baddeley *et al.* 1986; Cruz-Orive & Hunziker, 1986 for further details, including the list of requirements to be met). Provided intersection counts are made over the same length $L(r)$, then $S(v)/S(pm)$ represents the estimator of $S(v)/V(r)$ divided by that of $S(pm)/V(r)$.

Data handling

Estimates of $S(v)/S(pm)$ obtained from each region of each animal were used to calculate group means and their coefficients of variation (CV = standard deviation/group mean). Differences between bowel regions and between methods of estimating $S(v)/S(pm)$ were analysed by non-parametric statistical tests, Page's 'L' trend test for related samples (Page, 1963; Miller, 1975) and Friedman's method for randomised blocks (Sokal & Rohlf, 1981).

RESULTS

An example of the appearance of tissue boundary traces in transverse and in longitudinal/vertical sections is illustrated in Figure 2. The traces were made from the proximal region (i.e duodenum) of Animal 4.

Surface amplification factors due to villi estimated by vertical, longitudinal and

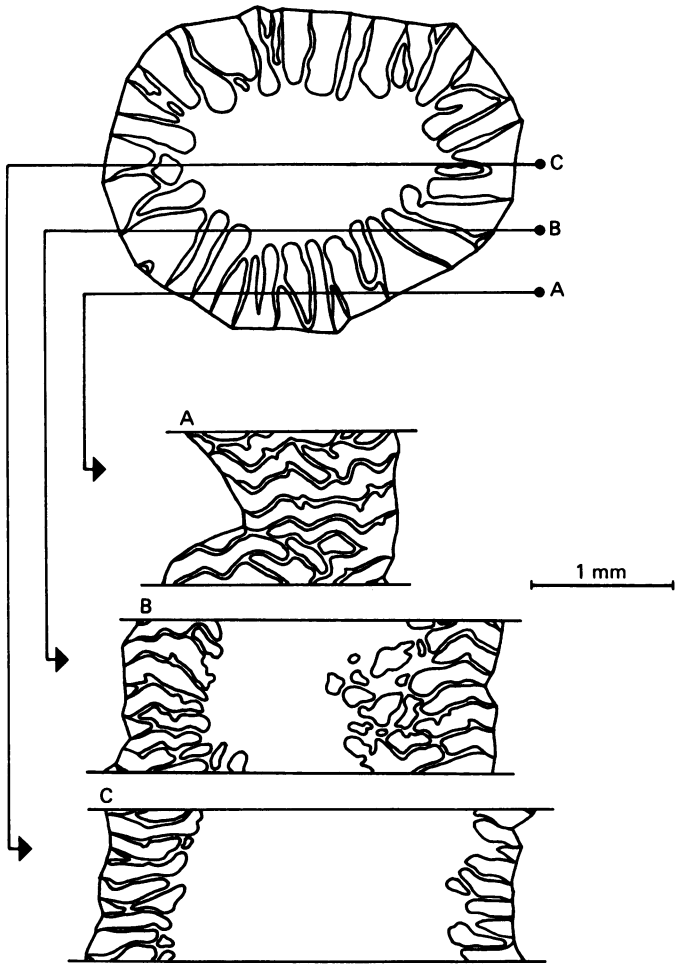

Fig. 2. Drawings of actual tracings from the duodenum of Animal 4. The top represents a transverse section on which the approximate positions of three systematic random vertical sections (A, B and C) are indicated. Section A is the most peripheral and C the most central of the set.

transverse sectioning approaches are given in Table 1. Values are shown for proximal, middle and distal bowel regions from each of six rats.

The results obtained using vertical sections and cycloid test lines reveal a gradient of surface amplification which declines from duodenum to terminal ileum. In duodenum, villi serve to amplify the mucosal surface area seven- to eight-fold but this figure falls to three- to four-fold in distal regions. The regional differences were significant (value of Page's 'L' = 82 for 3 regions and 6 rats; $P < 0{\cdot}01$).

Surface amplifications obtained by analysing longitudinal and transverse sections with straight test lines were not significantly different from those obtained by using vertical sections and cycloid test lines. Both model-based approaches reproduced the essential characteristics of the proximodistal gradient of villous amplification.

Table 1. *Surface amplifications due to villi in proximal, middle and distal regions of small bowel estimated by three different methods*

Bowel region	Animal	Surface amplification estimated by using: Vertical sections	Longitudinal sections	Transverse sections
Proximal	1	9·03	8·26	8·15
	2	5·40	6·81	8·38
	3	6·42	6·50	7·44
	4	6·82	7·92	6·58
	5	11·20	9·31	8·19
	6	6·54	6·36	6·53
	Group mean	7·57	7·53	7·55
	Observed CV	0·283	0·155	0·110
Middle	1	7·58	9·12	7·72
	2	9·19	8·71	6·85
	3	5·38	5·37	6·56
	4	10·25	11·80	6·48
	5	8·64	9·39	5·98
	6	6·01	5·45	6·53
	Group mean	7·84	8·31	6·69
	Observed CV	0·240	0·300	0·087
Distal	1	1·89	1·83	2·52
	2	5·12	5·90	2·98
	3	3·75	3·42	3·07
	4	3·36	3·55	3·92
	5	4·09	3·43	3·59
	6	3·09	3·65	3·71
	Group mean	3·55	3·63	3·30
	Observed CV	0·303	0·359	0·160

DISCUSSION

The present study has demonstrated that the principal assumptions underlying a geometric model of rat small bowel are capable of generating estimates of villous amplification factors essentially similar to those provided by vertical sectioning. Since estimates obtained by vertical sections + cycloid arcs are unbiased in general (given that the necessary requirements are satisfied, Baddeley *et al.* 1986), it is concluded that present model-based estimates exhibit negligible bias.

These findings imply that the geometric model proposed earlier (Mayhew, 1984) constitutes a satisfactory representation of biological reality. This is despite the facts that (*a*) individual villi display anisotropic surfaces and (*b*) local anisotropies exist due to the primary mucosal tube being non-circular and to the presence of Peyer's patches. The essential validity of the underlying assumption that the villous surface is *globally* isotropic seems to depend on two principal factors: villi are disposed radially (see Fig. 2, trace of transverse section) and their bases zig-zag around the perimeter of the primary mucosa (Fig. 2, section A). These factors are unlikely to alter during mucosal atrophy/hypertrophy because villi are relatively fixed in number throughout life (Clarke, 1972; Forrester, 1972). Therefore, it seems unlikely that trophic changes alone could destroy the global isotropy of villi during experimental fasting (Ross & Mayhew, 1985) and diabetes (Carson & Mayhew, 1987).

Given negligible bias, it is natural to enquire whether or not model-based estimates

can be obtained more efficiently than by using vertical sections. Taking values of CV^2 to approximate relative efficiencies, it appears from results (Table 1) that transverse sectioning is roughly six times more efficient than longitudinal or vertical sectioning. This comparison is misleading for two reasons. First, it is more appropriate to use the mean square error rather than the variance when assessing the efficiency of an estimator which is not unbiased in general. Second, the vertical sectioning approach employed here was not optimal. Opening the small bowel along its length, laying it flat on a substratum and taking section planes vertical to the substratum (see Baddeley *et al.* 1986, their Fig. 4) would provide a better vertical section design than the present one. Whether or not this improved design is more efficient than transverse sectioning remains to be tested. In view of the zig-zag arrangement of villous bases, it is possible that section-to-section variation would still be greater than that found with transverse sections.

Taken together, present findings justify the use of a model-based approach based on transverse sectioning for calculating villous amplification factors in rat small bowel (Mayhew, 1984; Mayhew & Middleton, 1985). The use of this model in past studies can now be justified on the grounds of negligible bias. Unfortunately, it is an inherent weakness of model-based approaches that they are not unbiased in general. In consequence, their validity must be tested on each and every occasion when they are invoked to tackle a new problem. Therefore, design-based methods are the method of choice unless negligibly-biased model-based methods of superior efficiency can be developed.

One final point: it is reassuring to note that global isotropy of villi in a structure so patently anisotropic as the small bowel does suggest that the assumption of isotropy may be reasonable in other situations (say, taking arbitrarily-orientated sections through renal glomeruli or spinal motoneurons) where it has yet to be validated by a design-based approach.

SUMMARY

Sampling schemes developed for use with a geometric model of rat small bowel are tested against a design-based scheme (vertical sectioning with cycloid test lines) which offers unbiased estimates of surface amplifications due to villi. The model-based methods comprise transverse and longitudinal sectioning coupled with putative correction factors.

Comparisons are based on proximal, middle and distal segments of six small bowels. Transverse and longitudinal sections through the same segments of each animal were analysed by conventional intersection counting (using straight test lines). Appropriate intersection ratios were multiplied by their respective correction factors in order to calculate surface amplifications. Longitudinal sections were employed further as vertical sections and intersections were counted with cycloid arcs to obtain unbiased estimates of surface amplifications.

Both model-based schemes (transverse and longitudinal) gave group mean values similar to those obtained by vertical sectioning. Therefore, the use of a geometric model in past studies on rat small bowel can now be justified on grounds of negligible bias.

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