

Riboflavin deficiency: early effects on post-weaning development of the duodenum in rats

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The aim of this present study was to identify the earliest point at which riboflavin deficiency affects post-weaning bowel development in rats. After weaning, eighty Wistar rats were weight-matched as pairs, one animal being fed a normal synthetic diet and the other being fed the same diet but deficient in riboflavin. Body weight, feeding and rates of growth were monitored and eight pairs of animals were taken for analysis at 45, 69, 93, 117 and 141 h. Riboflavin status was monitored by determining the erythrocyte glutathione reductase activation coefficient (EGRAC), and hepatic flavins were measured by a fluorescence assay. Changes to the number and dimensions of villi and crypts in the duodenum were determined, as well as crypt division (bifurcation) and the DNA synthesis index of the crypt epithelium by bromodeoxyuridine (BrdU) labelling. Riboflavin deficiency was established in the experimental rats, as demonstrated by a significant increase in EGRAC after 45 h ($P < 0.001$) and decreased liver flavins after 96 h ($P < 0.001$). After 96 h a significant increase in the size and cellularity of the crypts ($P < 0.001$ in both cases) was seen in these riboflavin-deficient animals, with a decreased incidence of bifurcating crypts and of BrdU-labelled cells. No changes to villus number or size were observed. The present study has demonstrated that developmental changes to the duodenal crypt arise shortly after circulating riboflavin measurements show evidence of deficiency. These changes primarily affect cell proliferation and crypt bifurcation, and precede long-term changes such as the reduction of villus number.

Riboflavin deficiency: Duodenum: Proliferation: Morphogenesis

The maturation of gastrointestinal function at the time of weaning is regulated in part by changes to the composition of the diet. Animal studies have identified qualitative and quantitative changes to the gastrointestinal tract following alterations in diet at this time. For example, weaning rats fed a diet low in carbohydrate but high in fat have a reduced expression of the brush-border enzyme sucrase, whereas the expression increases in those animals fed a diet high in carbohydrates but low in fat (Henning & Guerin, 1981). In lambs the transition from milk to grass results in a reduction in the concentration of monosaccharides in the lumen. As a consequence there is a very rapid reduction in the expression of the Na⁺/glucose co-transporter, but D-glucose and other glucose analogues infused into the lumen can rapidly restore expression of the Na⁺/glucose co-transporter (Dyer *et al.* 1997).

In rats, weaning is associated with a transient increase in the crypt cell proliferation in the small intestine and a

decreased expression of brush-border lactase. However, when fed a diet of reduced protein content, no increase in epithelial proliferation occurred and lactase continued to be expressed at preweaning levels (Buts & Nyakabasa, 1985). In rats fed an Fe-deficient diet post-weaning a significantly reduced activity of sucrase, lactase and maltase, and synthesis of secretory component were observed (Lanzkowsky *et al.* 1982; Buts & DeMeyer, 1984). Alteration to the fatty acid composition of the weaning diet was also found to change the transport of galactose, hexose and lipids (Thomson *et al.* 1989).

We have reported that dietary deficiency of riboflavin for a period as short as 7 d following weaning results in morphological and cell kinetic changes to the gastrointestinal tract of weaning rats (Williams *et al.* 1995, 1996a, b). After 7 d of riboflavin depletion crypt hypertrophy was evident, and there were fewer villi per unit area of mucosa compared with controls. After more prolonged depletion

Abbreviations: BrdU, bromodeoxyuridine; DBA, *Dolichos biflorus agglutinin*; EGRAC, erythrocyte glutathione reductase activation coefficient.

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villus hypertrophy was observed and may have represented an adaptation response to this deficiency. In this current study, cell kinetic and morphological changes to the duodenal epithelium were measured 2–6 d following weaning in rats fed either a normal diet, or a diet deficient in riboflavin. The aim of the present study was to identify the earliest point at which riboflavin deficiency affects post-weaning duodenal development in the rat.

Materials and methods

Materials

All buffer salts, chemicals and histological dyes were purchased from Sigma Chemical Ltd (Poole, Dorset, UK) and from BDH Gurr (Poole, Dorset, UK). The primary antibody for bromodeoxyuridine (BrdU) immunostaining was purchased from Dako Ltd (Ely, Cambs., UK) and the secondary antibodies and detection reagents from Vector Laboratories (Peterborough, Cambs., UK). Reagents for measuring glutathione reductase and liver flavins were purchased from Sigma Chemical Ltd. All dietary components were purchased from Sigma Chemical Ltd, with the exception of arachis oil (Hillcross Pharmaceuticals Ltd, Burnley, UK), Briggs salt mixture (Seaford Laboratories, Seaford, East Sussex, UK) and acid-washed casein (MRC Dunn Unit, Cambridge, UK).

Animals

Wistar rats were bred in pathogen-free isolated conditions and were housed in wire-bottomed cages to prevent coprophagy. They were permitted free access to tap water and maintained under standard laboratory conditions with a 12 h light–dark cycle, a mean temperature of 21°C and a mean humidity of 45%. All procedures were carried out in compliance with the current UK Home Office Regulations and under project license PPL 50/1309.

Dietary protocol and weight matching

Eighty female weaning Wistar rats weighing between 40 and 65 g were used for the study. Animals were weaned from their mothers on postnatal day 21. On arrival rats were paired by weight and allocated to one of two dietary groups. One rat from each pair was assigned to the riboflavin-deficient group and fed a semi-synthetic diet prepared in-house containing no added riboflavin. The depleted diet contained (g/kg): 30 arachis oil, 700 sucrose, 200 casein 0.24 riboflavin-free vitamin mixture, 50 Briggs salt mixture. The residual content of riboflavin in this diet was determined as 0.52 mg/kg diet. This is sufficient to allow growth to continue and to prevent overt clinical signs of deficiency. The other rat of the pair received a control diet consisting of the same semi-synthetic diet but further supplemented with 15 mg riboflavin/kg.

The inanition effects of a diet deficient in riboflavin are well known (Parsons & Dias, 1991; Williams *et al.* 1996a). To allow study of the effects of riboflavin deficiency *per se* on gastrointestinal development, which is also influenced by body weight (Younoszai & Ranshaw, 1974), weight

matching was carried out. The animals fed the riboflavin-deficient diet were fed *ad libitum* throughout the study, but the control animals were fed an amount of diet sufficient to maintain their weight equal to that of the riboflavin-deficient partner ($\pm 10\%$). To achieve matching the rats were weighed and fed daily between 09.00 and 10.00 hours.

Study design

The rats were maintained on their respective diets for 45, 69, 93, 117 or 141 h from weaning to death. These times reflect the arrival of the animals from the breeding colony in the afternoon to the point at which the animals were killed. At each time point, eight weight-matched pairs of animals were killed by chloroform inhalation.

Tissue sampling

At 60 min before exanguination each animal received an intraperitoneal injection of 0.5 ml BrdU (0.4 mg/g body weight prepared in isotonic saline (9 g NaCl/l)). After exanguination, the thorax was opened and blood was collected by cardiac puncture. Erythrocytes were separated, washed in isotonic saline and stored in distilled water at a ratio of 1:3 (v/v) at -20°C . The liver was also removed, rinsed in saline, weighed and frozen at -20°C .

Determination of erythrocyte glutathione reductase activation coefficient

The activity of erythrocyte glutathione reductase was measured using the method described by Glatzle *et al.* (1970) and modified (Powers *et al.* 1983) for use on the Cobas Bio Autoanalyser (Roche Diagnostics, Welwyn Garden City, Herts., UK). The activity of the enzyme was measured in the venous haemolysate as activity with: activity without exogenous FAD. An erythrocyte glutathione reductase activation coefficient (EGRAC) greater than 1.3 was taken to indicate unsaturation of the enzyme with FAD and evidence of biochemical deficiency (Tillotson & Baker, 1972).

Liver flavins

Liver flavins were measured using a fluorimetric assay according to principles described by Bessey *et al.* (1949). The liver samples were homogenized, the flavins extracted, and the fluorescence measured using a Perkin Elmer 3000 (Boston, MA, USA) fluorescence spectrometer (Perkin Elmer, at 450 nm excitation and 510 nm emission wavelengths). Background fluorescence was measured by the addition of sodium dithionite to reduce riboflavin to the non-fluorescent dihydroriboflavin. Total flavins were calculated after a 37°C overnight incubation. The two sets of fluorescence values were then used to calculate concentrations of FAD, FMN and riboflavin, and total liver flavins as $\mu\text{g/g}$ liver wet weight. As FMN and riboflavin fluoresce with the same intensity it is not possible to separate their relative contributions to the overall fluorescence, hence they were measured together.

Histology

The small intestine was carefully dissected, rinsed in saline and its length recorded. Two 10 mm segments were cut from the pyloric sphincter representing the duodenum. The first segment was fixed in 4% (v/v) formaldehyde in PBS (pH 7.4) cut into small transverse segments and bundled in 3M tape (Potten & Hendry, 1985). The tissue bundle was then dehydrated, cleared in xylene, embedded in Paraplast wax and 5 μ m thick serial sections cut on a rotary microtome (Anglia Scientific, Cambridge, UK). The histologist was 'blind' to the identity of samples for all histological analyses.

Scanning electron microscopy and counts of villus number

Sections of intestine were opened longitudinally, pinned out with the luminal surface facing up, and fixed in 1% (v/v) glutaraldehyde in 0.1M-sodium cacodylate buffer overnight. The tissue was then washed, treated with 1% (v/v) OsO₄, dehydrated, critically point dried (Polaron 3000, East Grinstead, UK), mounted on Al stubs, and sputter-coated in Au (Edwards S150 Edwards High Vacuum International, Crawley, W. Sussex, UK). Electron micrographs of the luminal surface were taken at 80 \times magnification on a Philips SEM501 scanning microscope (Philips, FEI Company Electron Optics, Eindhoven, The Netherlands). An acetate grid of defined area was placed over each micrograph and villi number per unit area determined. Five randomly-placed grids were counted on each micrograph per rat. The number of villi per unit area was then expressed as mean villi/mm².

Counts of crypt bifurcation

Bundles of intestine were prepared as described previously. These were sectioned transversely (5 μ m) and stained with haematoxylin and eosin to determine the incidence of crypt bifurcation. Four non-serial sections from each tissue block were analysed per animal, and the total number of crypts and number of crypts with clear evidence of splitting at their base (bifurcation), were counted around the circumference. Only those crypts in which a plane of division extended for several cell positions upwards from the Paneth cells were counted. It is likely that this method underestimates the number of bifurcating events, since only those crypts dividing perpendicular to the plane of sectioning will be observed. However, these considerations apply equally to control and riboflavin-deficient groups. Bifurcation was expressed as a percentage of total crypts per section.

Crypt and villus dimensions

The measurements were made with four non-serial 5 μ m transverse sections from each animal. The sections were stained with haematoxylin and eosin, and viewed and measured with an image analysis system (Image Manager, PC; Sight Systems, Worthing, West Sussex, UK) calibrated (in μ m) using an ocular magnification of 10 \times . A total of forty crypts and twenty villi were measured from each animal. To measure the crypt height only those crypts in which the lumen was present from the base to the mouth of

the crypt (i.e. centrally sectioned) were measured. Villus height was measured where the *lamina propria* core was present from the base to the tip of the villus.

Immunostaining for bromodeoxyuridine

This procedure was carried out according to the method described by Wynford-Thomas & Williams (1986). Sections were denatured in 1M-HCl for 3 min at 60°C, neutralized in 0.2M-boric acid and 0.05M-disodium tetraborate (pH 8.4), washed in PBS and stained with a mouse monoclonal anti-BrdU antibody (Dako Labs, Glostrup, Denmark) at 1/750 dilution. The immunostain was detected by avidin-biotin peroxidase ABC kit (Vectastain; Vector Laboratories) with diaminobenzidine substrate. The sections were counterstained in 1 μ g Hoechst 33342 dye/ml, dehydrated, cleared and mounted in DPX (BDH Gurr). For each animal the total cell number and number of labelled cells per crypt (only centrally-sectioned crypts were chosen) were counted in four non-serial sections. The labelling index was then expressed as % BrdU-labelled crypt cells.

Statistical analysis

A two-way ANOVA followed by a Scheffe test was used to investigate effects of time and diet on all independent variables that were normally distributed, which were final body weight, food consumption, g weight gain/g diet, liver flavin concentrations, crypt depth, incidence of crypt bifurcation, villus number, villus length and BrdU-labelling index. For EGRAC, which was not normally distributed, the Kruskal-Wallis ANOVA was applied, followed by the Mann-Whitney *U* test.

Results

Riboflavin status and food consumption

Erythrocyte glutathione reductase activation coefficient. The measurement of EGRAC established that in the groups

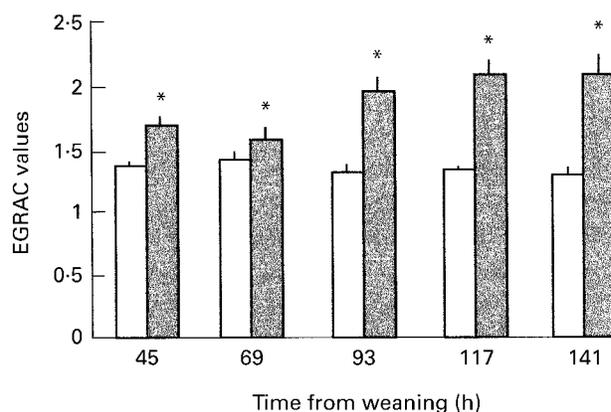


Fig. 1. Measurement of erythrocyte glutathione reductase activation coefficient (EGRAC) in rats maintained on a normal (□) or a riboflavin-depleted (■) diet from weaning for 45, 69, 93, 117 or 141 h. Values are means with their standard errors represented by vertical bars for eight weight-matched pairs of animals. Mean values were significantly different from those for the control group (Kruskal-Wallis and Mann-Whitney *U* test): **P* < 0.05. For details of diets and procedures, see p. 594.

Table 1. Liver flavin concentrations in rats fed either a riboflavin-deficient (RD) or control (C) diet from weaning†
(Means with their standard errors for eight weight-matched pairs of animals)

Time on diet (h)	Liver FAD ($\mu\text{g/g}$ wet wt)				Liver FMN + riboflavin ($\mu\text{g/g}$ wet wt)				Total liver flavins ($\mu\text{g/g}$ wet wt)			
	C		RD		C		RD		C		RD	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
45	18.9	1.20	15.03*	0.83	3.88	1.04	2.96	0.42	22.83	1.26	18.04*	0.96
69	14.72	0.96	12.19	1.04	4.32	0.50	3.02*	0.24	19.06	0.81	16.02	1.44
93	17.73	0.52	13.61**	0.76	4.56	0.26	3.13**	0.15	22.09	0.91	16.66***	0.75
117	19.09	0.49	13.34***	0.52	4.84	0.23	2.97***	0.11	23.22	0.66	16.17***	0.58
141	24.77	0.92	15.28***	0.62	7.90	0.39	4.46***	0.25	32.71	1.11	19.73***	0.72

Mean values were significantly different from those for the controls (ANOVA followed by scheffe test): * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

† For details of diets and procedures, see p. 594.

fed a riboflavin-deficient diet there was a progressive increase in the activation coefficient for the enzyme, consistent with depletion of riboflavin (Fig. 1). By 45 h the difference between the groups had reached significance ($P<0.01$) and remained so for the duration of the experiment.

Liver flavin concentrations. A decrease in hepatic concentrations of FAD, FMN and riboflavin, as well as total liver flavins, was observed in the animals fed the riboflavin-deficient diet. The decrease in FAD had reached significance by 45 h (Table 1), and by 141 h had fallen to only 60% of the levels found in the livers of control animals ($P<0.0001$).

Growth and food consumption. The food consumption was not significantly influenced by time on the diet (Table 2). Consistent with the weight-matching regimen there was no overall difference in the mean body weight, or increase in body weight between the control and riboflavin-deficient diet groups, except at 93 and 117 h where the animals fed a deficient diet weighed slightly less (7.4% and 8.1% respectively). Weight gain (g/g diet) was lower in the animals on the riboflavin-deficient diet after 117 h and remained low at 141 h.

Gastrointestinal morphology and cytokinetics

Villi number and length. No effect of time, or diet, on the unit density (villi/mm²) or height of the villi was observed (Table 3).

Crypt depth and bifurcation. An increase in the height of the crypts in animals fed the riboflavin-deficient diet was evident after 69 h (Fig. 2), and this difference was maintained throughout the experiment ($P<0.001$). Crypt bifurcation in riboflavin-deficient animals failed to increase with time from weaning, an effect which was seen in control animals (Fig. 3). From 93 h the proportion of crypts undergoing crypt bifurcation was significantly lower in riboflavin-deficient animals ($P<0.01$).

Crypt cellularity. No increase in crypt cellularity was observed in the control group (Fig. 4), in contrast with animals fed the riboflavin-deficient diet. This increase was significant after 69 h ($P<0.001$).

Crypt cell proliferation. In contrast to control animals the proliferative BrdU index fell from 69 h in the animals fed the riboflavin-deficient diet, and values remained significantly lower than those for the control animals at all time points thereafter ($P<0.001$; Fig. 5).

Discussion

The present study has demonstrated in the post-weaning rat that morphological and kinetic changes to the duodenal epithelium can be observed after 2 d in those animals fed a riboflavin-deficient diet. The critical change observed in the present study affected the multiplication of the intestinal crypts that contain the epithelial stem cells, transit amplifying cells and differentiating enterocytes, goblet, Paneth and enteroendocrine cells. In the rats fed the

Table 2. Growth and food consumption of rats fed either a riboflavin-deficient (RD) or a control (C) diet from weaning†
(Mean with their standard errors for eight weight-matched pairs of animals, except at 0 h, where values are for forty animals)

Time on diet (h)	Body wt at kill (g)				Food consumed (g/d)				Wt gain (g/g diet)			
	C		RD		C		RD		C		RD	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
0	48.93	1.16	47.24	1.17	—	—	—	—	—	—	—	—
45	55.04	1.89	53.77	1.88	11.84	0.60	13.02	0.45	0.19	0.02	0.14	0.02
69	64.44	2.49	61.14	5.62	—	—	12.63	0.82	0.19	0.02	0.17	0.01
93	50.79	0.91	47.06*	0.85	13.81	0.71	11.94	0.97	0.17	0.01	0.15	0.01
117	62.02	0.91	56.98*	0.89	14.10	0.54	14.34	0.72	0.22	0.01	0.17*	0.01
141	62.19	2.20	56.92	2.28	15.64	1.22	17.32	0.42	0.19	0.01	0.13*	0.01

Mean values were significantly different from those for the controls (ANOVA followed by Scheffe test): * $P<0.05$.

† For details of diets and procedures, see p. 594.

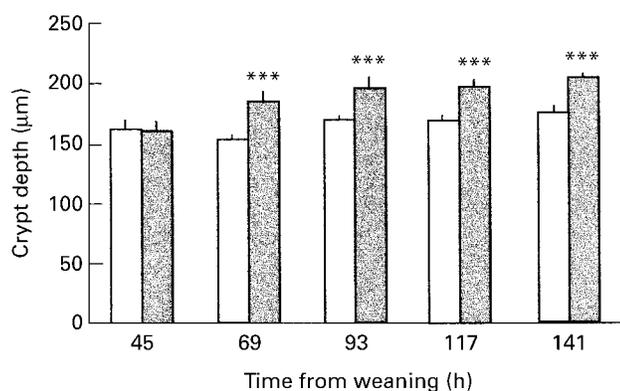
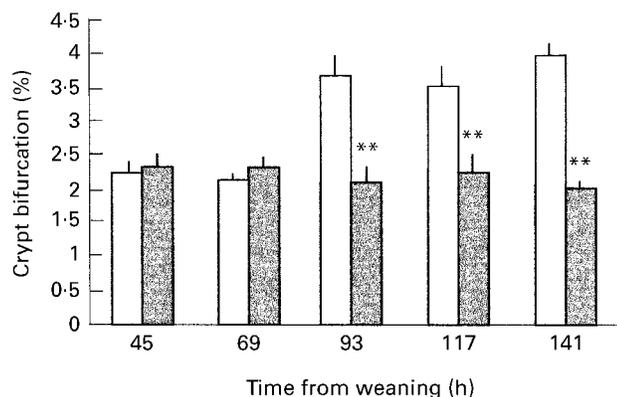
Table 3. Effect of feeding a riboflavin-deficient (RD) or control (C) diet to rats from weaning on the length of villi and density of villi*

(Means with their standard errors for eight weight-matched pairs of animals)

Time on diet (h)	Villus length (μm)				Villus density (μm)			
	C		RD		C		RD	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
45	573.6	13.0	561.6	13.6	NA		NA	
69	583.8	11.2	613.7	10.7	NA		NA	
93	590.4	15.3	629.3	9.2	57.0	1.7	57.5	3.3
117	599.4	10.2	584.0	29.2	58.7	2.7	54.3	3.0
141	622.3	18.8	599.3	12.3	62.2	2.1	61.7	2.0

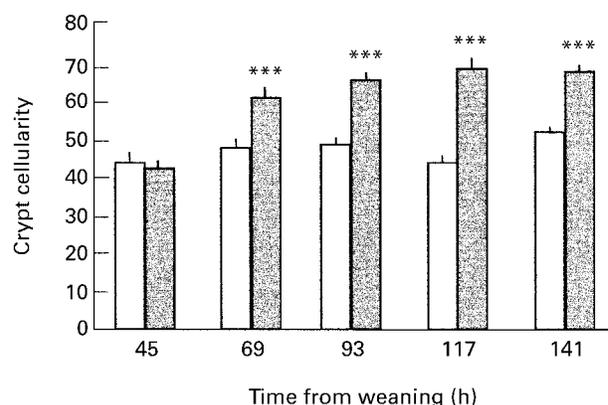
NA, values for the 45 and 69 h villus density were not obtained due to problems with tissue handling.

* For details of diets and procedures, see p. 594.

**Fig. 2.** Measurement of crypt depth in rats maintained on a normal (□) or a riboflavin-depleted (■) diet from weaning for 45, 69, 93, 117 or 141 h. Values are means with their standard errors represented by vertical bars for eight weight-matched pairs of animals. For each animal the depth of twenty centrally-sectioned crypts were measured. Mean values were significantly different from those for the control group (ANOVA and Scheffe test): *** $P < 0.001$. For details of diets and procedures, see p. 594.**Fig. 3.** Measurement of crypt bifurcation in rats maintained on a normal (□) or a riboflavin-depleted (■) diet from weaning for 45, 69, 93, 117 or 141 h. Values are means with their standard errors represented by vertical bars for eight weight-matched pairs of animals. For each animal the incidence of bifurcating crypts was determined in four non-serial sections for each animal. Mean values were significantly different from those for the control group (ANOVA and Scheffe test): ** $P < 0.01$. For details of diets and procedures, see p. 594.

riboflavin-deficient diet crypt depth and cellularity increased, but the BrdU-labelling index and proportion of crypts bifurcating decreased. The present study has added to existing evidence that riboflavin deficiency post-weaning can influence the development of the duodenum in rats. No change to the villus population was observed in this present study, suggesting that the crypt population is the target of the earliest effects of riboflavin deficiency. This finding is consistent with those of other studies in which changing levels of hormones, or diet, were shown to impact on the crypt stem cells. Our previous studies (Williams *et al.* 1995, 1996a, b) examined the effects of this deficiency for longer periods after weaning, and showed that riboflavin deficiency restricts the expansion of the duodenal villus number. The present study has demonstrated that these changes to the villus population probably follow as a consequence of earlier effects on the crypt epithelium.

These changes to the crypts are significant in the context of bowel development, but also in the process by which the stem cells are regulated. It has been suggested that the

**Fig. 4.** Measurement of relative crypt cellularity in rats maintained on a normal (□) or a riboflavin-depleted (■) diet from weaning for 45, 69, 93, 117 or 141 h. Values are means with their standard errors represented by vertical bars for eight weight-matched pairs of animals. For each animal the number of cells from the base to the neck of centrally-sectioned crypts were determined in twenty crypts for each animal. Mean values were significantly different from those for the control group (ANOVA and Scheffe test): *** $P < 0.001$.

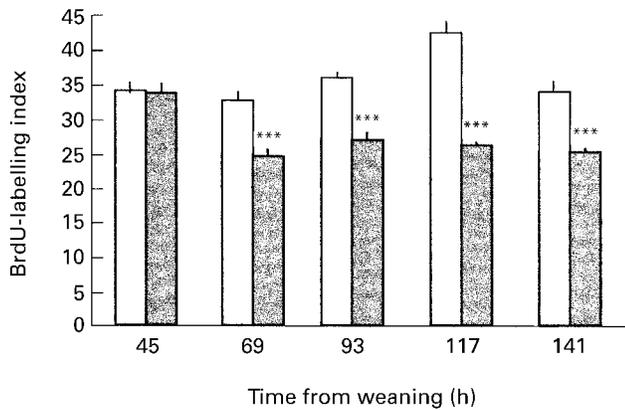


Fig. 5. Measurement of the bromodeoxyuridine (BrdU) proliferative-labelling index by immunohistochemistry in rats maintained on a normal (□) or a riboflavin-depleted (■) diet from weaning for 45, 69, 93, 117 or 141 h. Values are means with their standard errors represented by vertical bars for eight weight-matched pairs of animals. For each animal the total number of crypt cells and of BrdU-positive cells in twenty centrally-sectioned crypts was determined in four non-serial sections for each animal. Mean values were significantly different from those for the control group (ANOVA and Scheffe test): *** $P < 0.001$.

regulation of crypt cellularity, size and bifurcation are tightly regulated and related to the control of the stem cell population (Totafurno *et al.* 1987; Potten & Loeffler, 1990). Studies by Ponder *et al.* (1985) have used mouse aggregation chimeras between strains expressing *Dolichos biflorus agglutinin* (DBA) lectin-binding sites and strains that do not. They observed during postnatal bowel development that crypts were polyclonal structures containing a mixture of DBA-positive and -negative cells. Later in development these crypts became monoclonal, either fully expressing or not expressing this marker. This finding suggested a process by which one type of cell, either DBA-positive or -negative, was removed, resulting in crypts containing a homogeneous population of cells. It was also observed that patches of DBA-negative crypts appeared, suggesting that they had expanded by a process of crypt bifurcation. The control of bifurcation may also involve a feedback mechanism such that crypts normally divide when their cellularity reaches a certain upper size limit (Totafurno *et al.* 1987).

Riboflavin deficiency post-weaning did not completely inhibit this process, but the post-weaning increase in crypt bifurcation seen in the control animals was not observed in those fed the riboflavin-deficient diet. The increase in crypt bifurcation in the control animals was consistent with other measures of proliferation and growth, and probably stimulated by physiological drive on the change to a solid diet. These changes included an increase in the proportion of proliferating cells (see Fig. 5) but not of crypt depth (see Fig. 2). Thus, in the normal rat as crypt proliferative activity increased, crypt bifurcation also increased, maintaining the crypt cellularity and depth. In contrast, in those animals fed the riboflavin-deficient diet there was no post-weaning increase in bifurcation, resulting in an increased crypt cellularity and depth. The depths of the crypts measured in the control animals were consistent

with those of similarly-aged animals (Goodlad & Wright, 1990), but the increased crypt depths in the riboflavin-deficient group exceeded these measurements. It might be expected that this increase in crypt cellularity would be matched by a faster migration of cells onto the villi. Whilst this factor was not examined in the present study, rats kept deficient for longer periods demonstrated an increase in the size of crypts and villi, and in the crypt cell production rate. This finding suggests that there is an altered kinetic balance in the duodenum of riboflavin-deficient animals. These present results are consistent with evidence that the precocious expression of brush-border hydrolases, induced by administration of a hydrocortisone injection to 9-d-old rats, requires an effect on the proliferative precursor crypt cell population (Henning *et al.* 1975).

Whilst no change to the villus population was seen in this present study, rats made riboflavin deficient for longer periods following weaning have a reduced density of villi compared with their weight-matched controls (Williams *et al.* 1995). In addition, the villi and crypts were enlarged compared with the controls. This developmental change was not reversible (Williams *et al.* 1996a), suggesting the crypts and villi became larger as a compensatory mechanism to increase the mucosal absorptive area.

Post-weaning increases in crypt and villus number have been reported and are consistent with the increasing size of the bowel during this period. However, some researchers have argued that the number of villi is laid down in late fetal and early postnatal development, and is then fixed after this point (Clarke, 1972; Forrester, 1972). In these other studies villi were counted along the entire small intestine and by different techniques. We have used scanning electron microscopy preparations so that the density of intact villi was directly determined, avoiding the stereological problems associated with counting villi in thin sections.

At the beginning of the present study animals were carefully weight-matched in pairs, and the feeding regimen of control animals was continually modified to match consumption by the riboflavin-deficient animal (see Table 1). Nevertheless, the rate of weight gain was decreased in those rats fed the riboflavin-deficient diet, consistent with the requirement for this vitamin in the utilization of energy from food (Olpin & Bates, 1982a, b). Using this experimental design it was possible to show that riboflavin deficiency was responsible for the developmental changes, and not other factors such as altered food consumption or body weight. Further evidence was the significant increase in EGRAC values after 48 h ($P < 0.05$), which was followed 24 h later by the first detectable changes to the crypts (see Fig. 2). Whilst the EGRAC values for both groups of animals were greater than 1.3 (i.e. conventionally regarded as normal; Tillotson & Baker, 1972) other researchers have demonstrated that EGRAC values may reach 1.5 even in rats fed riboflavin-replete diets (Powers *et al.* 1983, 1991; Duerden & Bates, 1985). However, consistent with the establishment of a riboflavin deficiency, reduced liver flavin levels (the marker of long-term deficiency), were observed after 96 h.

Whether the effect of riboflavin deficiency on crypt bifurcation is direct, or indirect, the present study has demonstrated that developmental changes arise first in the

crypts shortly after measures of circulating riboflavin status are altered. Further studies have now shown that if rats are kept riboflavin replete by intramuscular injection of FMN, changes to the crypts still occur if the animals are fed a diet deficient in riboflavin (CA Yates, GS Evans and HJ Powers, unpublished results). This finding suggests that a crypt luminal-sensing mechanism may be involved in the response to dietary riboflavin deficiency.

Riboflavin deficiency is endemic in many regions of the world (Bamji, 1981; Powers *et al.* 1985; Brun *et al.* 1990). Exposure to a riboflavin-deficient environment *in utero*, and postnatally, may compromise human duodenal development through similar mechanisms to those proposed here.

Acknowledgement

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