Acid microclimate in coeliac and Crohn's disease: a model for folate malabsorption

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SUMMARY The surface pH of human proximal jejunum was measured in biopsy samples and found to be more acid than the phosphate buffer in which they were incubated. The *in vitro* jejunal surface pH was 5·93 ± 0·05 in control subjects and 6·19 ± 0·09 in treated coeliac patients. A group of untreated coeliac patients with a surface pH of 6·56 ± 0·14 had a significantly less acid surface pH compared to controls, as did a group of Crohn's patients with a surface pH of 6·21 ± 0·04. These two groups with a significantly raised surface pH were subdivisible into 'high' and 'low' groups. Surface pH was found to remain low in the treated coeliac and control groups but became more acid over the incubation period reaching almost normal values in the Crohn's group and the untreated coeliac group. There were significant inverse correlations between villous and microvillous height with initial surface pH. The raised surface pH in untreated coeliac disease and Crohn's disease would alter the amount of a weak acid available for non-ionic diffusion. Therefore the present results may help to explain the folate malabsorption known to occur in untreated coeliac disease and the frequently seen low serum folate levels in Crohn's disease.

To account for deviations from the pH-partition hypothesis as applied to weak electrolyte transfer in the rat small intestine, Hogben and his colleagues (1959) proposed the existence of an acid microclimate on the surface of the gut. They argued that weak-electrolytes must be confronted with a region of acid pH lower than the measurable luminal pH if transfer were to be explained solely by non-ionic diffusion. Experiments in the rat with pH-microelectrodes (Lucas *et al.*, 1975; Lucas and Blair, 1978) have demonstrated that such a region of acid pH exists having a value of at least pH 5·5 when the bulk phase pH is 7·4. Preliminary experiments have similarly demonstrated a low surface pH in man using biopsy samples from the proximal jejunum (Lucas *et al.*, 1976; Cooper *et al.*, 1977). Alterations in the pH of this microclimate have been suggested (Blair and Matty, 1974) as a cause of the folic acid malabsorption seen in coeliac disease (Cooke, 1968). The frequent occurrence of folate deficiency in Crohn's disease (Hoffbrand *et al.*, 1968; Swan,

1969; Eade *et al.*, 1972) which is seen even when the jejunum is apparently uninvolved might also be associated with a deficient acid microclimate. For this reason pH-electrodes were used to measure the pH at the surface of the jejunum in biopsy samples from normal subjects, patients with treated and untreated coeliac disease and in patients with Crohn's disease who have apparently normal proximal jejunum. The purpose of the present experiments was to measure the surface pH in these various groups and to correlate the jejunal surface pH with any alterations in jejunal morphology.

Methods

ELECTRODE CONSTRUCTION AND CHARACTERISTICS

The construction of pH-microelectrodes based on the method of Portnoy (1967) has been described elsewhere in detail (Lucas and Blair, 1978). They consisted of 1 mm o.d. inert capillary glass having a pH-sensitive membrane across one end, forming a moderately convex surface, projecting approximately 35 μm beyond the inert capillary glass. All electrodes gave a linear response over the pH 4-9 range of not less than 55 mV per decade and

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had a resistance of $4 \times 10^9 \ \Omega$; 95% of the maximal response was evident within 30 seconds.

BIOPSY PROCEDURE
Peroral jejunal biopsies were taken with a suction biopsy capsule (Roy-Choudhury et al., 1964) from control subjects and patients after an overnight fast. Samples were taken immediately for electrode experiments and measured by an experimenter unfamiliar with the criteria of gastroenterological diagnosis, which prevented unintentional bias. The delay between obtaining samples and immersion in buffer medium was less than one minute.

CLINICAL NOTES

Normal subjects and coeliac patients
Jejunal biopsies were taken on the basis of informed consent after an overnight fast and at the same time of day. The age and sex composition of the groups under consideration did not vary significantly. Control population surface pH did not alter with age of subject and therefore alterations in mean age and sex of the groups were not a significant source of error. Normal tissue was obtained from 19 subjects (10 men and nine women, mean age 36 years) who were either normal volunteers (range 13-75 years) (eight subjects) or patients who had absolutely normal jejunal histology and no demonstrable gastrointestinal disorder. Coeliac tissue was obtained from nine patients (five men and four women, mean age 42 years, range 14-68 years) before starting any treatment, including a gluten-free diet and from nine patients (five men and four women, mean age 46 years, range 27-73 years) who had been on a gluten-free diet for periods between one and 10 years.

Crohn's patients
Fifteen adult patients (seven men and eight women, mean age 37 years, range 21-70 years) with Crohn's disease which was diagnosed according to conventional criteria (Linder et al., 1963; Lockhart-Mummary and Morson, 1964) were selected randomly from patients on the basis of a radiologically normal jejunum.

The length of history of the disease varied from one year to 17 years. Of the patients, nine had involvement of the colon alone, and six had involvement of the terminal ileum either alone (three) or in addition to colonic involvement (three). No specific features of Crohn's disease were found in the jejunal biopsies except for raised plasma cell counts in the lamina propria, confirming that all patients had no apparent jejunal involvement.

Ten patients had had no bowel resection at any time and five patients had had one bowel resection each (one ileocaecal resection, one colectomy with ileorectal anastomosis, and three panproctocolectomy with ileostomy). Three patients were on steroids at the time of biopsy (one tablet ACTH 5μ daily, two tablets prednisolone 40 mg and 20 mg daily respectively, and two patients were taking salazopyrin). At the time of biopsy 11 patients had indices showing active disease (disease activity is indicated by haemoglobin less than $12 \cdot 5$ g/μ, serum albumen less than 40 g/l, and seromucoids greater than $1 \cdot 5$ g/l); four had normal indices.

ELECTRODE MEASUREMENT PROTOCOL
Jejunal samples of adequate size were pinned mucosal surface upwards in 10 ml cork-buttomed flasks containing Krebs phosphate buffer gassed with 100% O_2 at 37°C. Added to the buffer was 10 mM glucose, a concentration known to cause maximal rates of jejunal acidification in the rat (Blair et al., 1975). Previously calibrated electrodes mounted on a Leitz micromanipulator were advanced on to the biopsy surface until the electrodes touched the villi and the whole preparation 'dimpled'.

The change in pH was recorded at one minute intervals over five minutes, during which time a steady pH reading was achieved. After this initial measurement the electrode was taken off the surface and the buffer pH rechecked. This procedure was repeated again after one hour, giving a surface pH reading, pH, initially (t_0) and after 60 minutes (t_{60}).

LIGHT MICROSCOPY
A piece of the jejunal biopsy was taken for routine histological examination. Sections $5 \ \mu$ thick were stained in haematoxylin and eosin, and villous height was measured using a standard technique (Roy-Choudhury et al., 1966).

ELECTRON MICROSCOPY
Electron microscopic examination was possible on the jejunum of 11 subjects including two coeliac patients on a normal diet and one coeliac patient on a gluten-free diet, three normal subjects, and five patients with Crohn's disease. Freshly excised biopsy material was fixed by immersion in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in Araldite resin. Representative sections were obtained from the villi, or from the luminal surface of material having a flattened mucosa, and examined on an AEI 801 electron microscope having a previously calibrated magnification scale.

STATISTICAL METHODS
Significance was tested using t tests for paired and

unpaired samples, as normal statistics are entirely appropriate for pH data (Lucas, 1977). As a check, the non-parametric Mann-Witney and Wilcoxon tests were applied but are not given here as no discrepancies were found using both these normal and non-normal statistics.

Results

INITIAL SURFACE pH
Surface pH was measured in all biopsy samples in the presence of 10 mM glucose at the onset of incubation in a Krebs-phosphate buffer of pH 6.89 ± 0.07 (52). The mean surface pH in all groups (Fig. 1, Table 1a) was significantly more acid than the buffer pH ($p < 0.001$ for all groups except untreated coeliac patients where the significance was $p < 0.02$). Significant differences in surface pH between the various groups became apparent on comparing them with the control surface pH of 5.93 ± 0.05 (19). The surface pH of 6.56 ± 0.14

(9) for the untreated coeliac group was significantly less acid ($p < 0.005$) than the control group, whereas, in contrast, the surface pH of 6.19 ± 0.09 (9) for the treated coeliac group was not. The surface pH for the Crohn's disease group of 6.21 ± 0.04 (15) was significantly ($p < 0.01$) less acid than the control group.

CHANGES IN SURFACE pH WITH TIME
The differences in surface pH between groups that were evident at the onset of incubation in the presence of glucose were not statistically significant at the end of the incubation period (Fig. 1, Table 1a). Although surface pH did not significantly change in the controls and in the tissues from treated coeliac patients, there was a significant move towards a more acidic surface pH in the previously less acidic Crohn's and untreated coeliac patients. This move towards more acid values was statistically significant in both groups.
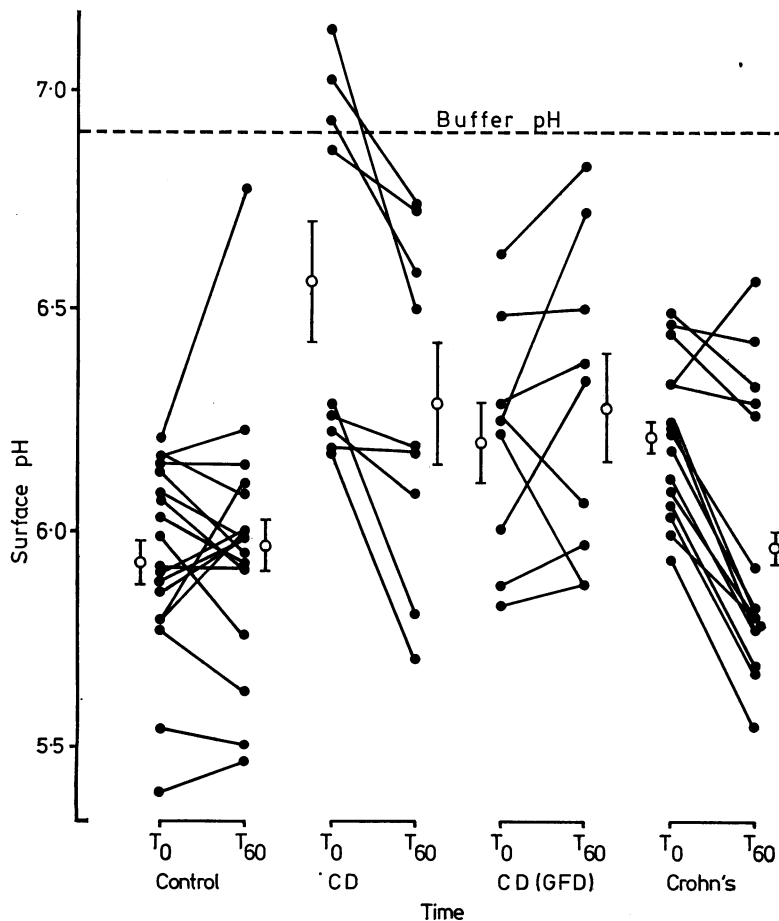

Fig. 1 *Surface pH in the presence of 10 mM glucose at the onset (t_0) and end (t_{60}) of incubation in control subjects, untreated coeliac patients (CD), treated coeliac patients (CD (GFD = gluten free diet)), and in Crohn's patients.*

Table 1a *Analysis of Fig. 1*

Buffer pH	t_0 6·89 ± 0·07 (52)		t_{00} 6·85 ± 0·03 (52)	
Surface pH	t_0	t_{00}	*Difference*	*Significance*
Controls (19)	5·93 ± 0·05	5·96 ± 0·07	−0·02 ± 0·04	NS
Coeliac disease (9)	6·56 ± 0·14	6·28 ± 0·13	0·29 ± 0·07	P < 0·005
Significance	P < 0·005	NS		
Coeliac disease (9) on GFD	6·19 ± 0·09	6·28 ± 0·12	−0·09 ± 0·08	P < NS
Significance	NS	NS		
Crohn's disease (15)	6·21 ± 0·04	5·96 ± 0·03	0·25 ± 0·04	P < 0·001
Significance	P < 0·01	NS		

Surface pH of proximal jejunum incubated at 37°C in phosphate buffer containing 10 mm glucose. Results expressed as mean ± SEM. Number of observations in parentheses.

Table 1b *Effect of 10 mm glucose on surface pH of proximal jejunum*

a *At onset of incubation*	*10 mM Glucose*	*No Glucose*[*]	*Difference*	*Significance*
Controls (8)	5·84 ± 0·10	6·28 ± 0·07[1]	−0·46 ± 0·09	P < 0·005
CD (6)	6·49 ± 0·21	6·79 ± 0·22[2]	−0·31 ± 0·10	P < 0·05
CD on GFD (7)	6·21 ± 0·11	6·42 ± 0·11[3]	−0·21 ± 0·07	P < 0·025
Crohn's (11)	6·16 ± 0·05	6·48 ± 0·05[4]	−0·28 ± 0·07	P < 0·02
At end of incubation				
Controls (8)	5·79 ± 0·09	6·67 ± 0·04[1]	−0·83 ± 0·09	P < 0·001
CD (6)	6·21 ± 0·13	6·90 ± 0·16[2]	−0·69 ± 0·10	P < 0·001
CD on GFD (7)	6·21 ± 0·14	6·65 ± 0·10[3]	−0·45 ± 0·13	P < 0·02
Crohn's (11)	5·85 ± 0·07	6·73 ± 0·05[4]	−0·82 ± 0·10	P < 0·001
[*]c *Difference in surface pH over incubation period in absence of glucose*				
ΔpH $(t_0$-$t_{00})$		*Difference*		
1. Controls (8)		−0·39 ± 0·07		P < 0·001
2. CD (6)		−0·11 ± 0·07		NS
3. CD on GFD (7)		−0·24 ± 0·10		P < 0·05
4. Crohn's (11)		−0·26 ± 0·06		P < 0·005

[*]a: Presence and absence of glucose at t_0. b: Presence and absence of glucose at t_{00}. c: Difference in surface pH over incubation period in absence of glucose. Details as for Table 1a.

RESPONSE TO GLUCOSE

Paired samples from the same biopsy enabled a comparison in the surface pH to be made in the presence and absence of glucose in all groups, at both the onset and end of incubation. The results in Table 1b for surface pH in the presence of glucose represent therefore subsets from the data presented in Table 1a and Fig. 1. A significant difference could be demonstrated in all groups between surface pH in the presence and absence of glucose. In the absence of glucose the surface pH was higher in all cases. This difference became greater at the end of incubation. This was partly because the untreated coeliac group and the Crohn's group became more acid when incubated in the presence of glucose and partly because, in the absence of glucose, most groups tended to drift towards a neutral surface pH.

This significant drift towards less acid values in the absence of glucose in most groups can be seen in Table 1b. Section (c) shows the mean differences in surface pH at the onset and end of incubation in the absence of glucose and was arrived at by subtracting the individual values that comprise the column in section (a) from those in section (b). There is a significant drift towards neutrality in the absence of glucose in every group except the untreated coeliac patients: this was because, in the absence of glucose the surface pH of this group is significantly raised even at the onset of incubation.

SURFACE pH IN ABSENCE OF GLUCOSE

The buffer pH at the onset and end of incubation is given in Table 1a and is representative for comparisons with all groups. At the beginning of incubation, with the exception of the untreated coeliac group there was a significantly more acid (P < 0·01) surface pH in all groups. This difference between bulk and surface pH in the absence of glucose was also evident (P < 0·02) at the end of incubation but only in the control and treated coeliac group.

SUB-DIVISION INTO HIGH AND LOW GROUPS

The individual results from both the Crohn's and untreated coeliac disease groups were subdivisible into two groups, 'high' and 'low' groups that were significantly distinct (Fig. 1). Both 'high' groups in untreated coeliac and Crohn's disease groups re-
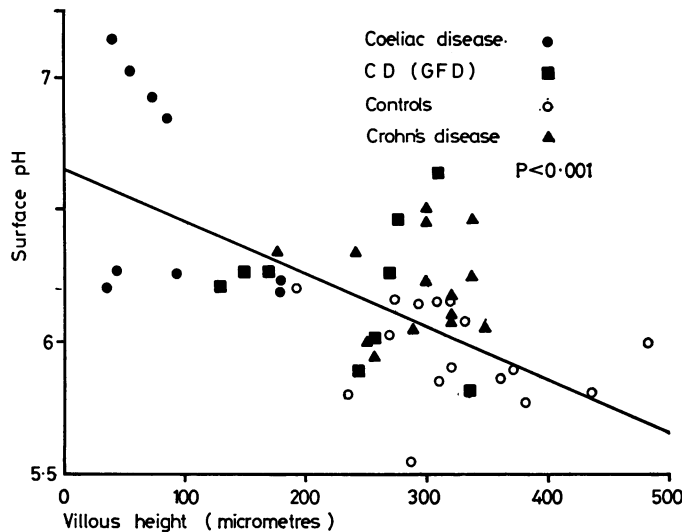
Fig. 2 *Correlation between villous height and surface pH at the onset of incubation in the presence of 10 mM glucose. Abbreviations as for Fig. 1.*

Table 2 *Subdivision of surface pH results from Table 1a for untreated coeliac and Crohn's disease into 'high' and 'low' groups*

Crohn's disease	t_0	t_{00}	Untreated coeliac disease	t_0	t_{00}
'High' (5)	6·41 ± 0·03	6·38 ± 0·05	'High' (4)	6·99 ± 0·06	6·63 ± 0·05
Significance v. control	P < 0·001	P < 0·001		P < 0·001	P < 0·001
'Low' (10)	6·11 ± 0·03	5·76 ± 0·03	'Low' (5)	6·23 ± 0·02	5·97 ± 0·12
Significance v. control	P < 0·05	NS		P < 0·001	NS
'High' v. 'Low'	P < 0·001	P < 0·001		P < 0·001	P < 0·001

Details as for Table 1a.

mained high over the incubation period; in contrast, both 'low' groups whose surface pH was still significantly higher than the control surface pH ($p < 0.05$) developed a final surface pH which was not significantly different from the controls. No such subdivision could be detected in the control or treated coeliac group (Table 2).

MORPHOLOGICAL AND OTHER FINDINGS
No significant correlations emerged between villous height and surface pH for the individual results within groups. When all results were considered there was a significant association (Fig. 2) between villous height and the surface pH at the onset of incubation in the presence of glucose; this association remained significant ($p < 0.05$) even when the four results from the 'high' group of untreated coeliac patients were discounted. From electron micrographs, microvillous height and glycocalyx depth was measured (Table 3). An inverse correlation (figure not shown) was found ($r = 0.65$, $p < 0.05$, n = 11) between surface pH and microvillous height—that is, the shorter the microvilli, the less acidic the surface pH. There was also a direct correlation ($r = 0.64$, $p < 0.05$, n = 10)

between fuzz depth and surface pH—that is, increasing fuzz depth was associated with a less acidic surface pH.

No significant correlations could be found between serum folate levels and surface pH for the individual results within any group: a general trend was visible when surface pH was correlated with mean serum folate levels for the four groups in which mean surface pH is inversely related to serum folate.

Discussion

Using the most frequently available samples of human gut tissue, the present *in vitro* experiments demonstrate that, when biopsy samples are incubated in phosphate buffer, differences are apparent between the pH measured at the jejunal surface and the pH measured in the bulk phase of the incubation medium. As with all *in vitro* methods, the relevance to *in vivo* circumstances must be examined. Two major differences exist between the *in vitro* incubation system and conditions *in vivo*: the buffer composition, and the fact that, in the interdigestive or fasting state *in vivo*, glucose enters predominantly from the serosal side through the basolateral

Table 3 *Morphological data and serum folate levels*

	Villous height (μm)	Microvillous height (μm)	Fuzz depth* (μm)	Serum folates ($\mu g/l$)
Controls	329 ± 15 (17)	1·16 (3)	0·07 (2)	6·50 ± 1·50 (10)
		(0·93, 1·3, 1·25)	(0·08, 0·06)	
Coeliac disease (normal diet)	87 ± 19 (9)	0·75 (2)	0·11 (2)	2·03 ± 0·44 (9)
		(0·84, 0·66)	(0·12, 0·09)	
Significance	P < 0·001			P < 0·02
Coeliac disease (gluten-free diet)	237 ± 24 (8)	1·25 (1)	0·11 (1)	4·23 ± 0·75 (8)
Significance	P < 0·01			NS
Crohn's disease	292 ± 12 (14)	1·51 ± 0·17 (5)	0·07 ± 0·01 (5)	3·91 ± 1·08 (14)
Significance	NS			NS

*Results are given as either mean ± SEM or as the mean with the individual results in parentheses under the mean.

enterocyte membranes.

Phosphate buffer was chosen to avoid the fluctuations in buffer pH seen in bicarbonate buffer in open systems, as it was important to stabilise the buffer pH. However, surface pH is unaffected by choice of phosphate or bicarbonate buffer (Lucas and Blair, 1978) in the rat: similarly, preliminary studies with biopsy samples in a bicarbonate containing buffer also show an acid surface pH (6·10 ± 0·11 (6)) similar to the results presented here. Although the biopsies were pinned out onto cork bases this was never sufficient to prevent serosal access of glucose: nevertheless *in vitro* the main route of glucose entry may be *via* the mucosally sited active pathway. The delay between obtaining fresh biopsy samples and their eventual immersion in buffer was minimal and indeed much less than most *in vitro* methods. Consequently, it is unlikely that the initial pH measurements were much affected by this change in the route of glucose access and it is difficult to envisage that freshly obtained biopsies had greatly differing initial intracellular glucose concentrations or that they could be depleted in the short time between biopsy and immersion in buffer.

In similar experiments in the rat (Lucas and Blair, 1978) an absence of glucose in the buffer caused a rise in the surface pH, as did metabolic inhibitors, anoxia, and the removal of sodium ions. In biopsy samples, the omission of glucose from the buffer causes a rise in the surface pH. Biopsies which were incubated for six hours or more, when it might be assumed that the tissue was undergoing autolysis, lost the ability to maintain the surface pH more acid than the buffer. These observations strongly suggest that the maintenance of an acid surface pH is dependent on the adequate *in vitro* functioning of the tissues. If an acid surface pH were to be caused by a moment of transient anoxia between biopsy and immersion, immersion in oxygenated buffer containing glucose should re-establish the originally neutral surface pH, which was not the case. Also the absence of substrate would lead to a loss of cellular function over incubation and if this were

the origin of the low surface pH, the surface pH would be more acidic in the absence of glucose. Since the surface pH was always less acidic in the absence of glucose in all groups studied and, further, that in the absence of glucose most groups became neutral on incubation, it was felt that the ability to demonstrate a significantly lower surface pH in the presence of glucose in all groups, both at the onset and end of incubation, adequately demonstrated the viability of the biopsy samples in all the groups concerned.

The apparent existence of subgroups within the two abnormal groups could reflect a difference in the extent of pathological change affecting the mucosa. In untreated coeliac disease, these subgroups may relate to similar variability in folic acid absorption (Cox *et al.*, 1958; Elsborg and Bastrup-Madsen, 1976). Within the subgroups in Crohn's patients, there was no correlation between surface pH and disease activity, site of macroscopic disease, length of history, treatment, or jejunal histology. Thus the variation in surface pH was not explained by other features of the disease.

A condition for the adequate maintenance of a low surface pH could be normal villous and microvillous architecture. Histological studies have shown an association between decreasing microvillous and villous height and a less acidic surface pH. Removing the Crohn's group, which is not characterised by a reduction in villous height, makes the correlation between surface pH and villous height more significant. This may mean that there is a failure to produce hydrogen ions which is compounded by the absence of villi in untreated coeliac disease that prevents pooling of hydrogen ions between villi, leading to an even greater rise in the surface pH. If the glycocalyx acts as a diffusion barrier to hydrogen ions (Blair and Matty, 1974), then thinning of the glycocalyx seen in untreated coeliac disease (Falchuk *et al.*, 1974) could cause a rise in surface pH. However, the predicted relationship between surface pH and glycocalyx thickness was not evident in the small sample examined and, in fact, the

reverse association was found. This may mean that qualitative rather than quantitative changes in the glycocalyx alter hydrogen ion diffusion.

Changes in surface pH in untreated coeliac disease and Crohn's disease over the incubation period in the presence of glucose are not simply related to a lower rate of glucose entry *in vitro* through the active pathway. A low rate of glucose entry, leading to a low rate of hydrogen ion production through metabolism of transported glucose, would only lower the surface pH to more acid values if the compartment in which surface pH is measured is closed off to the lumen—that is, if hydrogen ions were secreted into a closed system. The loss from this compartment, which is open to the lumen, depends on physical parameters only. Provided these do not change—for example, no change in the hydrogen ion diffusion coefficient or in the thickness of any retaining layer—a change in surface pH can only represent an increase in hydrogen ion production by the cells *per se* or achieved secondarily by increased glucose entry or metabolism. These surface pH changes point possibly to the absence *in vitro* of an inhibitory factor acting either on the system that produces hydrogen ion or in the input of glucose into that system in untreated coeliac and Crohn's disease.

A more acid pH on the jejunal surface would alter the concentrations of unionised forms of acids and bases immediately next to the enterocyte membrane such that the proportion of unionised form available for diffusion will differ completely from that in the bathing medium. Alterations in the surface pH would therefore affect the rate of transfer of weak electrolytes. As predicted by the pH-partition hypothesis, alkalinising the jejunum *in vivo* decreases the transfer of folic acid (Benn *et al.*, 1971; MacKenzie and Russell, 1976). The malabsorption of weak acids such as folic acid, as is frequently seen in untreated coeliac disease, is often explained in terms of the reduced surface area available for diffusion or by the reduced transfer of glucose, sodium ion, and fluid (Gerson *et al.*, 1974). However, an altered pH microclimate at the gut wall would explain weak acid malabsorption equally as well but would further explain why in partial villous atrophy, more quinine (pKa = 8·4) is absorbed than in healthy subjects (Mattila *et al.*, 1973), despite a presumed reduction in both surface area and glucose transfer capacity. The raised surface pH values presented here would help to explain the low serum folate levels seen in untreated coeliac disease and their resumption to normal values in treated disease. The present data also indicate an altered surface pH in Crohn's disease and imply a malabsorptive component to the low serum folate levels frequently seen in this disease. The altered surface pH in Crohn's disease adds to a growing body of evidence (Goodman *et al.*, 1976; Dunne *et al.*, 1977) which points to abnormalities in apparently uninvolved small bowel in this disease.

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