Small intestinal bacterial flora and folate status in gastrointestinal disease

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SUMMARY Serum folate levels and the bacteriology of the small intestine were studied in 13 patients with jejunal diverticulosis, 29 patients with partial gastrectomy, and five patients with ileal disease. The mean serum folate level in the patients with partial gastrectomy ($7.2 \text{ m}\mu\text{g/ml}$) was similar to that of control subjects but the mean level in the patients with jejunal diverticulosis ($14.6 \text{ m}\mu\text{g/ml}$) was significantly higher than the mean level of the control group ($8.0 \text{ m}\mu\text{g/ml}$). Five of the 13 patients with jejunal diverticulosis and two of the patients with partial gastrectomy had levels above the upper limit of the control group ($> 16.6 \text{ m}\mu\text{g/ml}$), and five of six patients with jejunal diverticulosis studied excreted raised amounts of folate in the urine ($> 13.2 \mu\text{g}$ in 24 hours). Serum folate in one of these patients with jejunal diverticulosis was identified chromatographically as 5-methyltetrahydrofolic acid. The mean serum folate level ($9.0 \text{ m}\mu\text{g/ml}$) in the patients with partial gastrectomy who had *Esch. coli* in the jejunal aspirates was significantly higher than in those without *Esch. coli* present (mean $4.2 \text{ m}\mu\text{g/ml}$). It is suggested that in gastrointestinal disease jejunal bacteria may contribute to the serum folate level even though this remains in an accepted normal range.

Some patients had low serum folate levels despite having a large number of *Esch. coli* in the jejunum. No evidence of malabsorption of folic acid was found in three such patients even though one had lactobacilli capable of consuming folate in the jejunum.

No relationship was found between serum folate level and ileal bacteriology.

It is well recognized that in patients with the intestinal stagnant-loop syndrome faecal microorganisms in the lumen of the small intestine may cause vitamin B_{12} deficiency by interfering with the absorption of dietary vitamin B_{12} . The relationship between the presence of these microorganisms and folate status in such patients is still uncertain. Some workers (Badenoch, Bedford, and Evans, 1955; Watkinson, Feather, Marson, and Dossett, 1959; Cooke, Cox, Fone, Meynell, and Gaddie, 1963; Barrett and Holt, 1967) have suggested that these microorganisms may cause folate deficiency by interfering with the absorption of dietary folate. Hoffbrand, Tabaqchali, and Mollin (1966), on the other hand, reported that faecal microorganisms in the upper small intestine may contribute to the body's folate stores by synthesizing folate within the lumen of the small intestine which is then absorbed.

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The present paper reports the results of a study of patients following partial gastrectomy, with jejunal diverticulosis, and with ileal disease, in whom the relation of serum folate level to the number, type, and distribution of bacteria in the small intestine is examined. In addition, the results of chromatographic studies to identify the form of folate circulating in patients with the intestinal stagnant-loop syndrome are reported.

Subjects Studied

The series included 25 healthy adult subjects, 29 patients with partial gastrectomy for benign peptic ulcer, including 17 who had undergone a Billroth I operation and 12 who had had the Polya type of operation. None of these patients had clinical or radiological evidence of small intestinal disease. There were 13 patients with multiple jejunal diverticulosis and five patients

with ileal disease, three of whom had radiological evidence of Crohn's disease; the remaining two had had an ileal resection for Crohn's disease.

All these patients had normal serum vitamin B₁₂ levels or were receiving regular maintenance vitamin B₁₂ therapy at the time of this study.

Methods

SERUM AND URINARY FOLATE LEVELS

These were measured by microbiological assay using *Lactobacillus casei* as described by Waters and Mollin (1961). Random 24-hour urine samples were collected into bottles containing 2 g of ascorbic acid for the urine folate estimations.

FOLIC ACID ABSORPTION

This was measured by the method of Chanarin, Anderson, and Mollin (1958) in which the patient receives an oral dose of folic acid (40 µg/kg body weight) after saturation with parenteral folic acid and the rise in serum folic acid concentration is determined microbiologically using *Streptococcus faecalis* as test organism. In normal subjects the peak rise is greater than 40 mµg/ml.

BACTERIAL PRODUCTION OR UTILIZATION OF FOLATE

The method used has been previously described (Hoffbrand *et al*, 1966). Essentially, bacteria were allowed to grow *in vitro* in a serum of measured folate (*L. casei*) content. At the end of the incubation period (usually 24 hours) the bacteria were removed by centrifugation and the folate content of the supernatant serum was measured again using *L. casei*.

BACTERIOLOGICAL STUDIES

Jejunal intubations were carried out on fasting subjects using a sterile open-ended tube. The tube was allowed to pass to the ileum in the five patients with ileal disease. The identification of bacteria was carried out by standard methods (Wilson and Miles, 1964) and quantitative bacterial counts were performed using the serial dilution method of Miles, Misra, and Irwin (1938). Anaerobic bacterial cultures were carried out as described by Gorbach, Nahas, Lerner, and Weinstein (1967).

CHROMATOGRAPHY OF SERUM FOLATE

Serum was diluted 1:5 in 0.02 M sodium-phosphate buffer, pH 6.1, containing 200 mg ascorbic acid per 100 ml and extracted by autoclaving at 121°C for two and a half minutes. Coagulated proteins

were separated by centrifugation and the clear supernatant was applied to a column (20 × 0.8 cm) of DEAE cellulose previously equilibrated with 0.02 M phosphate buffer, pH 7.0. The folate derivatives were eluted by a linear gradient of increasing phosphate concentration (pH 7.0) from 0.02 M to 0.5 M. Both buffers contained 0.2% mercapto-ethanol and eluted fractions of 5 ml were collected into tubes containing 0.1 ml of 1% (w/v) ascorbic acid. The folate content of each tube was measured microbiologically with *L. casei*.

The following substances were also chromatographed: (¹⁴C) labelled 5 methyltetrahydrofolic acid (5(methyl¹⁴C)H₄ Pte Glu), (³H) labelled folic acid (³H)Pte Glu, and pteroyltriglutamic acid (Pte Glu₃).

Results

SERUM FOLATE LEVELS

The serum folate levels in the control subjects and in the patients with partial gastrectomy, jejunal diverticulosis, and ileal disease are shown in Figure 1. In the control subjects, the serum folate levels ranged from 3.0 to 16.6 mµg/ml (mean 8.0 mµg/ml). Of the 29 patients with partial gastrectomy, two had serum folate levels above the upper limit of the control group, but the mean serum folate level of the 29 patients (7.2 mµg/ml) was not significantly different from that of the control subjects. On the other hand, of the 13 patients with jejunal diverticulosis as many as five had serum folate levels above the upper limit of the control group and the mean serum folate level

FIG. 1. The serum folate levels of the control subjects and of patients after partial gastrectomy, with jejunal diverticulosis, and with an ileal lesion.

of these 13 patients (14.6 m μ g/ml) was significantly greater than that of the control group ($P < 0.01$). None of the five patients with ileal disease had a raised serum folate level and their mean serum folate level was only 4.8 m μ g/ml.

URINARY FOLATE EXCRETION IN PATIENTS WITH JEJUNAL DIVERTICULOSIS

The 24-hour urine folate excretions in 10 of the normal subjects ranged from 0.7 to 13.2 μ g (mean 7.0 μ g). Five of six patients with jejunal diverticulosis tested excreted more than 13.2 μ g of folate in 24 hours and the mean excretion among the six patients, 24.9 μ g in 24 hours, was significantly raised ($P < 0.01$) (Table I).

Control Subjects			Jejunal Diverticulosis		
Subject	Serum Folate (m μ g/ml)	Urine Folate (μ g/24 hr)	Patient	Serum Folate (m μ g/ml)	Urine Folate (μ g/24 hr)
1	13.6	13.2	1	10.1	47.4
2	6.0	11.1	2	17.6	37.5
3	8.1	11.0	3	8.8	21.3
4	5.3	10.4	4	9.0	19.4
5	4.2	6.5	5	16.8	16.1
6	3.4	6.4	6	5.6	7.5
7	8.6	6.2			
8	3.2	3.7			
9	6.6	1.1			
10	5.3	0.7			
Mean	6.4	7.0		11.3	24.9

Table I The serum folate levels and 24-hour urine folate excretions of 10 randomly selected control subjects and six patients with jejunal diverticulosis

RELATION OF SERUM FOLATE LEVELS TO JEJUNAL BACTERIOLOGY

All the patients with jejunal diverticulosis had *Esch. coli* present in the jejunal aspirates in concentrations ranging from 10^4 to 10^9 organisms per ml. In patients following partial gastrectomy there was a striking difference in the serum folate level according to whether or not *Esch. coli* were present in the jejunal fluid (Fig. 2). Among the 11 patients without *Esch. coli* present, there was only a narrow range in serum folate level (from 2.2 to 6.3 m μ g/ml, mean 4.2 m μ g/ml) whereas there was a wide range in the serum folate levels (from 1.6 to 27.0 m μ g/ml) of the 18 patients who had had a gastrectomy, in whom *Esch. coli* were present in the jejunal fluid. The mean level of 9.0 m μ g/ml was significantly higher than that of the group without *Esch. coli* ($P < 0.01$). Nevertheless, there were three patients with *Esch. coli* present who had serum folate levels below the lower limit of the control range (< 3.0 m μ g/ml).

The relation between the serum folate levels of the patients with partial gastrectomy and jejunal diverticulosis and their quantitative counts of *Esch. coli* in the jejunal fluid is shown in Figure 3. There was no direct correlation, though high serum folate levels tended to occur only in patients with high counts of *Esch. coli*. A few patients, however, had low normal or subnormal serum folate levels despite having high counts of *Esch. coli*. Further studies were performed on these particular patients in an attempt to clarify the relationship between their serum folate levels and their jejunal bacteria.

FURTHER STUDIES IN PATIENTS WITH LOW SERUM FOLATE LEVELS AND HIGH JEJUNAL *Esch. coli* COUNTS

Absorption of folic acid

Folic acid absorption studies were carried out in three of these patients in order to determine

FIG. 2. The serum folate levels in the patients after gastrectomy divided according to whether or not *Esch. coli* were present in the jejunal aspirate.

FIG. 3. The serum folate levels in the patients after gastrectomy and with jejunal diverticulosis related to the quantitative *Esch. coli* counts in the jejunal aspirate.

whether the low serum folate levels could be attributed to malabsorption of folate. The results are shown in Table II. Folic acid absorption was normal in all three patients and the absorption did not change significantly in one patient after treatment with broad-spectrum antibiotics

(tetracycline 1 g daily in divided doses for seven days) which reduced the jejunal bacterial counts from 10^9 to 10^3 organisms/ml.

BACTERIAL FOLATE PRODUCTION AND UTILIZATION *in vitro*

The jejunal bacteria from one of these patients (case 1, Table II) were cultured and tested for folate production and utilization *in vitro*. The principal organisms isolated were *Esch. coli* (10^9 /ml) which were found to produce folate, and a *Lactobacillus* (10^8 /ml) which consumed folate (Table III). A third organism frequently found in high concentrations in the jejunal juice of the patients with the intestinal stagnant-loop syndrome was bacteroides. This organism, isolated from a patient with jejunal diverticulosis and a high serum folate level, like *Esch. coli*, synthesized folate (Table III).

RELATION OF SERUM FOLATE LEVEL TO ILEAL BACTERIOLOGY

High *Esch. coli* counts in the ileum, unlike high counts of *Esch. coli* in the jejunum, did not appear to be associated in any patient with a raised

Case	Diagnosis	Serum Folate Level (µg/ml)	Jejunal Bacteriology (count/ml juice)	Folic Acid Absorption (µg/ml)			
				0	1	2	3
1	Partial gastrectomy	1.6	<i>Esch. coli</i> (1×10^9)	0	84	81	50 ¹
			<i>Lactobacilli</i> (1×10^7)	1	67	42	33 ²
2	Partial gastrectomy	2.3	<i>Esch. coli</i> (2×10^8)	1	61	75	30
3	Jejunal diverticulosis	4.7	<i>Esch. coli</i> (2×10^9)	0	112	101	80

Table II The serum folate levels, jejunal bacteriology, and absorption of folic acid in two patients following partial gastrectomy and one patient with jejunal diverticulosis

¹Before antibiotic therapy.

²After antibiotic therapy.

Case	Organism	Incubation			Free Folate Activity (µg/ml)	
		Medium	Time (hr)	Atmosphere	Before	After
1 Partial gastrectomy	<i>Esch. coli</i>	Serum	24	Aerobic	0.2	7.5
	<i>Lactobacilli</i>	Lactobacilli broth	24	Aerobic	2.4	1.4
4 Jejunal diverticulosis		Lactobacilli broth + pteroylglutamic acid	24	Anaerobic	21.8	1.5
	<i>Esch. coli</i>	Serum	24	Aerobic	0.8	3.2
	<i>Bacteroides</i>	Serum	48	Anaerobic	1.1	5.0

Table III The production and utilization of (*L. casei* active) folate by the jejunal bacteria of one patient following partial gastrectomy (case 1, Table II) and one patient with jejunal diverticulosis

Case	Diagnosis	Serum Folate Level (µg/ml)	Jejunal Bacteriology (Esch. coli count/ml juice)	Ileal Bacteriology (Esch. coli count/ml juice)
5	Resection	8.2	10 ⁸	10 ⁸
6	Crohn's disease	5.9	10 ⁸	10 ⁸
7	Resection	4.4	0	10 ⁸
8	Crohn's disease	2.7	0	10 ⁸
9	Crohn's disease	2.6	0	4.5 × 10 ⁷

Table IV The serum folate levels and Esch. coli counts in jejunal and ileal aspirates from the five patients with ileal lesions

serum folate level (Table IV). In the five patients in whom ileal intubations were carried out, the counts ranged from 4.5×10^7 to 1×10^8 /ml but the serum folate levels were all in the low normal or subnormal range.

IDENTIFICATION OF SERUM FOLATE IN A PATIENT WITH JEJUNAL DIVERTICULOSIS

Figure 4 compares the elution pattern after DEAE column chromatography of serum folate from a patient with jejunal diverticulosis and raised serum folate level with that of a normal subject, and with the elution pattern of standard marker solutions of 5-methyltetrahydrofolate, pteroyltriglutamic acid, and pteroylglutamic acid. The serum from both subjects showed a single peak, microbiologically active for *L. casei* and not for *S. faecalis*, which coincided with the peak of radioactivity after chromatography of radioactive 5-methyltetrahydrofolic acid. No other folate compounds were detected in the sera.

Discussion

The results of these studies confirm previous

FIG. 4. The results of DEAE column chromatography of serum folate from a normal subject and from a patient with the stagnant-loop syndrome due to jejunal diverticulosis compared with solutions of radioactive 5-methyltetrahydrofolate, radioactive folic acid (pteroylglutamic acid), and non-radioactive pteroyltriglutamic acid. The activities of the nonradioactive compounds in the different fraction numbers are expressed in terms of growth of *L. casei* (measured turbidimetrically) whereas the activities of the radioactive compounds are expressed as counts per minute (measured in an Inter technique liquid scintillation counter).

observations (Hoffbrand *et al*, 1966) that high serum folate levels may be found in some patients with faecal bacteria in their upper small intestine. They also show that raised urine folate excretion occurs in such patients. This is consistent with the theory that these high serum folate levels are due to excess production of folate by the intestinal bacteria and not to reduced urinary excretion of the vitamin.

Normal serum folate has been identified as 5-methyltetrahydrofolic acid (Herbert, Larrabee, and Buchanan, 1962). Previous studies have shown that the folate compound in the serum of patients with the intestinal stagnant loop syndrome is active for *L. casei* but not for *S. faecalis* (Hoffbrand *et al*, 1966). This suggested that the compound was either a pteroyltriglutamate or 5-methyltetrahydrofolate. The present studies show that in a patient with jejunal diverticulosis and a raised serum folate level, the folate compound circulating behaves chromatographically as well as microbiologically as 5-methyltetrahydrofolate.

The present studies also show that faecal bacteria in the jejunum may contribute to the patient's serum folate level even though this remains within an accepted normal range, a phenomenon previously suggested by Klipstein and Samloff (1966). The evidence for this is as follows: 50% of postgastrectomy patients with *Esch. coli* organisms in the jejunum had serum folate levels in a normal range but above the upper limit of the serum folate in those postgastrectomy patients without *Esch. coli*, and the mean serum folate in those with bacteria was significantly higher than in those without.

There was a poor correlation both in patients with a partial gastrectomy and in patients with jejunal diverticulosis between quantitative counts of *Esch. coli* in the jejunum and serum folate level but there are several possible reasons for this. It may be that the bacterial count does not give an accurate estimate of the total number of bacteria in the jejunum. Also different strains of *Esch. coli* may synthesize folate at different rates (Klipstein and Lipton, 1970). Moreover, microorganisms other than *Esch. coli* may be involved in folate synthesis, for instance bacteroides which may be present in very high concentrations in some patients with the stagnant-loop syndrome. Indeed, other organisms present may, as shown here, consume folate. The patient's dietary folate intake may also profoundly affect the serum folate level. Finally, the height of the serum folate level may not be a good indication of the total folate produced by the bacteria since the

rate of clearance of folate from plasma may well vary from one patient to the next, and indeed in the small number of patients studied here, there was a poor correlation between serum folate and urine folate excretion.

A few of the patients studied here with large numbers of faecal bacteria in the jejunum had extremely low serum folate levels. Barret and Holt (1966) reported one such patient and suggested that jejunal bacteria interfered with folic acid absorption, since in their patient folic acid absorption, initially subnormal, improved after antibiotic therapy. Wakisaka (1968) also found malabsorption of folic acid in four patients with the intestinal blind-loop syndrome which improved to normal after antibiotic therapy. In one patient studied here organisms (lactobacilli) were present in the jejunum which were capable of consuming folic acid *in vitro*. In the three patients with high *Esch. coli* counts and subnormal serum folate levels studied here, however, including the patient in whom lactobacilli capable of consuming folate were demonstrated, folic acid absorption tests were normal and in one patient folic acid absorption was not altered by antibiotic therapy. These findings do not exclude the possibility that bacteria in these patients interfere with the absorption of dietary folate, since the amount of folic acid used to test absorption was unphysiological and may have 'swamped' the bacteria. Moreover, food folate consists mainly of pteroylpolyglutamates, not of folic acid, and it will therefore be necessary to test the absorption of a physiological dose of pteroylpolyglutamates in such patients before a firm conclusion can be reached on this point.

Finally, the present results show that bacteria in the distal small intestine do not affect folate status. This is not surprising since folate is poorly absorbed from the ileum in man (Hepner, Booth, Cowan, Hoffbrand, and Mollin, 1968).

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(³H) Labelled folic acid and (¹⁴C) labelled 5-methyltetrahydrofolic acid were obtained from the Radiochemical Centre in Amersham and pteroyltriglutamic acid was given by Professor M. Friedkin.

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