Availability of monoglutanamyl and polyglutamyl folates in normal subjects and in patients with coeliac sprue

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SUMMARY Intestinal folate absorption was assessed in six normal subjects and in four patients with coeliac sprue who were studied before and after treatment by dietary gluten exclusion. Comparisons were made of the luminal disappearance from the perfused jejunum of $^3$H-pteroylmonoglutamate and pteroyl $^{14}$C-glutamylhexaglutamate, and of the 48-hour urinary recovery of each isotope after perfusion and a tissue saturating dose of folic acid. The labelled urinary folates consisted of folic acid, 10-formyltetrahydrofolate, and 5-methyltetrahydrofolate. In each group urinary recovery of $^3$H was significantly greater than that of $^{14}$C, confirming the evidence from jejunal perfusion that the availability of monoglutanamyl folate is greater than that of polyglutamyl folate. According to the urinary recovery data, both folates were poorly absorbed in untreated coeliac sprue, but were normally absorbed after treatment. Assuming uniform displacement of the absorbed labelled folates by the parenteral flushing dose, the finding of greater urinary isotope recovery than of luminal folate disappearance from the perfused proximal jejunal segment suggests an adaptation of the distal small bowel for folate absorption in coeliac sprue.

The nearly universal finding of folate deficiency in adult patients with untreated coeliac sprue (Weir, 1974) reflects the fact that the proximal small intestine, the optimal site of absorption of folic acid (Hepner et al., 1968; Halsted et al., 1971), is most severely affected in this mucosal disease. Dietary folates are a mixture of pteroylpoliglutamates (Butterworth et al., 1963) which, during the process of intestinal absorption, are hydrolysed to pteroyl-monoglutamate (PteGlu) by an intestinal mucosal $\gamma$-carboxypeptidase known as folate conjugase (Rosenberg et al., 1969; Butterworth et al., 1969; Baugh et al., 1971; Halsted et al., 1975). Using the technique of jejunal perfusion of separately labelled $^3$H-pteroylmonoglutamate ($^3$H-PteGlu) and pteroyl $^{14}$C-glutamylhexaglutamate ($^{14}$C-PteGlu$\gamma$), we recently described extreme malabsorption of both compounds in untreated coeliac sprue (Halsted et al., 1977).

Assessment of small intestinal function from data obtained by jejunal perfusion is limited to the perfused intestinal segment, and thus may overlook adaptive responses in the more distal bowel. Previous studies have likened coeliac sprue to a jejunal resection, in that increased intestinal uptake of glucose and electrolytes could be demonstrated in the perfused ileum of certain patients (Silk et al., 1975). A previous perfusion study in man showed that the normal intestinal uptake of PteGlu decreased as more distal segments were tested, whereas the uptake of PteGlu from the upper and lower jejunum was similar in four patients with coeliac sprue (Hepner et al., 1968). Providing that the absorbed labelled folate is uniformly displaced from the tissues by a flushing dose of parenteral folic acid, measurement of urinary isotope recovery can be used as an index of total intestinal folate absorption. In the present report, comparisons are made of folate absorption assessed by the two methods of jejunal perfusion and of urinary isotope recovery in normal subjects and in patients with coeliac sprue. The data suggest that the lower small intestine adapts to decreased jejunal folate absorption in coeliac sprue.
The urinary folate excretion data confirm our previous evidence (Halsted et al., 1977) that 3H-PteGlu is more available than 14C-PteGlu\textsubscript{7} in health and in mucosal disease.

**Methods**

**Patients**

Urinary isotope recovery was measured after jejunal folate perfusion in six normal adult male volunteers and in four adult patients with coeliac sprue who were studied both in exacerbation and in partial remission. These subjects were included in the larger groups from which jejunal perfusion data have previously been reported (Halsted et al., 1977). The normal volunteers were hospital or professional personnel, aged 28 to 53 years. The patients with coeliac sprue included two men and two women aged 43 to 58 years, each of whom had suffered from diarrhoea for more than a 20 year period, with associated weight loss or inability to gain weight. Three of the patients were studied before treatment, and again after a six to eight week period of gluten-free diet. The fourth patient was initially studied while in remission and again after 10 days of gluten challenge (two slices of whole wheat bread per day). Laboratory findings before treatment or after gluten challenge included low d-xylene absorption (Roe and Rice, 1948) (five-hour urinary excretion of 1.8 g to 3.5 g after an oral dose of 25 g; normal more than 4.5 g) steatorrhoea (Van de Kamer et al., 1949) (25.3 g to 48.0 g faecal lipid per day; normal less than 7 g), and a typical flat jejunal mucosal specimen obtained by Crosby capsule biopsy. In remission, every jejunal mucosal biopsy showed partial villous atrophy, while five-hour d-xylene excretion (3.3 to 5.6 g) and faecal lipid excretion (8.4 to 14.0 g/day) had improved in each case, with weight gain and decreased stool frequency.

As previously described (Halsted et al., 1975), folate absorption was studied using two separately labelled compounds, 3H-PteGlu (3’5’9 [3H]-folic acid, Amersham/Searle) and 14C-PteGlu\textsubscript{7}. The latter compound was synthesised by the solid phase method (Krumdieck and Baugh, 1969), so that the 14C label was on the first glutamyl unit (provided by Professor C. L. Krumdieck, University of Alabama) and hence remained in the folic acid molecule after hydrolysis. One litre jejunal perfusion solutions of isotonic saline were prepared containing 4 \( \mu \text{mol} \) (2 \( \mu \text{mol} \)) of 3H-PteGlu and 2 \( \mu \text{mol} \) (2 \( \mu \text{mol} \)) of 14C-PteGlu\textsubscript{7}. The solutions were perfused at a site 10 cm distal to the ligament of Treitz at a rate of 10 ml/min. After a 45 minute period of equilibration, samples were collected at a rate of 1 ml/min from a site 30 cm downstream. On completion of the perfusion, each subject received an intramuscular, tissue-saturating ‘flushing’ injection of folic acid (Folvite, Lederle Laboratories, Pearl River, NY), 34 \( \mu \text{mol} \) (15 mg). The jejunal perfusion tube was then withdrawn and urine was collected for the next 48 hours for measurement of isotope recovery. The collections were made in four consecutive 12 hour periods. Maximal isotope recovery occurred in the first 12 hour periods, with less than 5% of the total recovery in the last 12 hour periods. Isotope counting was performed on a Beckman LS 260 liquid scintillation counter, with efficiencies of 29.2% (3H) and 62.0% (14C). One 12 hour urine collection from a normal subject was made in the presence of 17 mmol (3 g) of ascorbic acid and then stored at \(-70^\circ\text{C}\). Subsequently, 10 ml of this collection were diluted with 200 mmol/\text{l} \(\beta\)-mercaptoethanol to a volume of 250 ml. The mixture (pH 6.4) was then applied to a DEAE-cellulose column, 20 \( \times \) 1 cm, and folates were eluted by a gradient constructed from 100 ml of phosphate buffer, 5 mmol/l, in a closed mixing chamber, attached to a reservoir of phosphate buffer, 500 mmol/l. The buffers were pH 6 and contained 200 mmol/l \(\beta\)-mercaptoethanol. Fractions were counted for radioactivity and assayed for folate activity using Lactobacillus casei and Streptococcus faecalis (Tamura et al., 1972). The identity of each peak was verified by chromatographing a separate urine sample with a variety of unlabelled and 3H-labelled authentic folate standards (Shane and Stokstad, 1976).

Previously described formulas (Halsted et al., 1975) were used to calculate luminal disappearances of each labelled folate (%/30 cm of perfused jejunal segment). Urinary recovery of each labelled folate in 48 hours was calculated as a percentage of that perfused. In expressing the relationship between luminal disappearance and urinary recovery, linear regression lines were computed by the method of least squares with a calculation for variance (Steele and Torrie, 1960). In this analysis, the absorption of 14C-PteGlu\textsubscript{7} was considered on the same slope as that of 3H-PteGlu, since present evidence indicates that 14C-PteGlu, produced by prior intestinal hydrolysis, is the form in which perfused 14C-PteGlu\textsubscript{7} is absorbed (Halsted et al., 1977; Dhar et al, 1977). The relationship between luminal disappearance and urinary recovery in coeliac sprue was compared in patients studied both before treatment, with flat jejunal biopsies, and in the same patients in early remission whose jejunal biopsies showed partial villous atrophy. The paired \( t \) test was used to compare results of absorption of each folate within each group, while the unpaired \( t \) test was used to compare results among the groups.
Results

Figure 1 depicts the 48 hour urinary recovery of $^3$H and 14C. In the normal group, and in each coeliac sprue group, the urinary recovery of $^3$H was significantly greater than that of 14C ($p < 0.001$ in each group). The recovery of each folate in the untreated coeliac sprue group was significantly less than that in the normal group ($p < 0.01, p < 0.01$), whereas recovery of each folate in the treated group was similar to that in the normal subjects.

Figure 2 shows the elution profile of labelled folate derivatives in urine. The elution positions of authentic folate standards, chromatographed under identical conditions and indicated by the arrows, are: A: 10-formyltetrahydrofolate; B: $^3$H p-amino-benzoylglutamate; C: 10-formylfolic acid; D: 5-formyltetrahydrofolate; E: 5-methyl-tetrahydro-($^3$H)-folate; F: tetrahydro-($^3$H)-folate, and G: folic acid.

Figure 3 shows the linear regressions of urinary isotope recovery in the control group and in the coeliac sprue patients studied before and after treatment. In the control group, the slope of the linear regression of 1.23 ± 0.27 indicated somewhat less than complete urinary recovery of radioactivity after absorption from the perfused jejunal segment. On the other hand, the finding of slopes less than 1.0 in each coeliac group, each significantly less than the slope of the control group ($p < 0.05, p < 0.005$), suggested greater folate absorption, and hence urinary isotope recovery, from the more distal small bowel than from the perfused jejunal segment in these patients.
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Analysis of folate absorption from measurement of recovery of urinary radioactivity supposes that all the excreted labelled compounds are folate derivatives. As demonstrated in Fig. 2, each label was excreted as a mixture of intact folates, the majority as $^3$H-PteGlu and $^{14}$C-PteGlu. Identical $^3$H and $^{14}$C labelling of these compounds is consistent with previous evidence that $^{14}$C-PteGlu$_7$ is absorbed as its hydrolytic product $^{14}$C-PteGlu, and hence enters the same metabolic pool as $^3$H-PteGlu (Rosenberg, 1976; Halsted et al., 1977). Apart from unmetabolised folic acid, the major folate metabolites in the urine were 10-formyltetrahydrofolate and 5-methyltetrahydrofolate. Others have described both these forms in the urine of scurvy patients (Stokes et al., 1975) and 5-methyltetrahydrofolate as the major urinary folate in normal subjects given flushing doses of parenteral folic acid (Chanarin and McLean, 1967; Godwin and Rosenberg, 1975).

The present study of folate absorption measured by urinary isotope excretion confirms our previous observations from jejunal perfusion studies that the availability of $^3$H-PteGlu is significantly greater than that of $^{14}$C-PteGlu, in normal subjects and in patients with coeliac sprue (Halsted et al., 1975, 1977). These data agree with those of Godwin and Rosenberg (1975) who described in normal subjects significantly greater mean urinary isotope excretion after oral $^3$H-PteGlu than that after oral $^{14}$C-PteGlu, each dose followed by a 34 $\mu$mol (15 mg) flushing dose of folic acid. The present technique has the advantage of the simultaneous administration of both folates, hence avoiding potential variations which may occur with sequential testing. By measuring changes in serum and urine levels after sequential oral doses and the haematological response to each folate form, Perry and Chanarin (1968) also described significantly greater availability of monoglutamyl than of polyglutamyl folate in normal subjects and in patients with megaloblastic anaemia. These findings contrast with those of Tamura and Stokstad (1973) who described similar folate levels in urine after the administration of oral doses of synthetic PteGlu and PteGlu$_7$ to normal volunteers in a steady state of folate saturation.

The present comparisons of urinary isotope recovery could reflect differences in experimental conditions between the groups, since, at the time of the flushing dose of parenteral folic acid immediately following perfusion, a greater proportion of labelled folate had been absorbed in the control group than in the patients with coeliac sprue. Several different studies have evaluated the effect of the flushing dose on tissue displacement of orally administered labelled folates. Calculations based on the change in serum levels and urinary excretion of folate one hour

![Fig. 3 Linear regression analysis of luminal disappearance of $^3$H-PteGlu and $^{14}$C-PteGlu$_7$ (\% on 30 cm perfused jejunal segment) and 48 hour urinary isotope recovery of $^3$H and $^{14}$C (percentage of administered amount). Closed symbols represent $^3$H compounds and open symbols represent $^{14}$C compounds. Each point represents a single study. The slope for the control group was significantly greater than the slopes obtained for each coeliac sprue group ($r < 0.05$, < 0.005).

Discussion

Previous studies, using oral doses and measurements of serum levels and of urinary excretion, described malabsorption of PteGlu in the majority of patients with untreated coeliac sprue (Anderson et al., 1960; Cooke et al., 1963; Stewart et al., 1967; Freedman et al., 1973). Hoffbrand et al. (1970) used microbiological serum folate assays in finding equally poor absorption of folic acid and conjugated folate in their coeliac sprue patients. On the other hand, using the method of jejunal perfusion of separately labelled monoglutamyl and polyglutamyl folates, we recently showed marked impairment of absorption of both folate forms in patients with untreated coeliac sprue, with incomplete recovery of jejunal luminal folate disappearance after clinical remission (Halsted et al., 1977). In the present analysis, folate absorption was measured by urinary isotope recovery in order to assess absorption from the entire intestine rather than just from the perfused jejunal segment. The data shown in Fig. 1 confirm the finding of impaired absorption of both monoglutamyl and polyglutamyl folate in untreated coeliac sprue, but show that, with treatment, correction of intestinal folate absorption is more rapid than previously suggested (Halsted et al., 1977).
after intravenous PteGlu, 68 μmol, suggested that the amount of folate taken up by the tissues was within the estimated range of total body stores (Halsted et al., 1967). Freedman et al. (1973) found reproducible daily urine excretion of radioactivity when normal subjects were tested each day for five days with oral ³H-PteGlu followed immediately each time by a parenteral flushing dose of 34 μmol. Two additional studies demonstrated that, within limits, the timing of the flushing dose is not critical to subsequent recovery of urinary isotope. Anderson et al. (1960) showed that the urinary excretion of radioactivity in normal subjects was not affected by varying the time of the parenteral flushing injection of 34 μmol from two hours before to two hours after the oral administration of ³H-PteGlu. In the studies of Godwin and Rosenberg (1975) urine isotope recovery was similar whether the tissue the flushing dose was absorbed at different rates, the flushing dose resulted in uniform displacement of the labels from the tissues.

A study of ileal mucosal histology and assessment of vitamin B₁₂ absorption indicated that the ileum is usually spared in coeliac sprue (Stewart et al., 1967). Subsequent perfusion studies showed heightened uptake from the ileum of perfused glucose, water, and electrolytes (Silk et al., 1975) and loss of the normal decreasing gradient for folic acid absorption between upper and lower jejunum in certain patients with coeliac sprue (Hepner et al., 1968). The present data comparing jejunal uptake and urinary isotope recovery suggest a distal intestinal adaptive response for absorption of both PteGlu and PteGlu₇ in coeliac sprue. In the absence of such a response, urinary isotope recovery should be less than the measured luminal disappearance, as occurred in the control group (Fig. 3). The patients with coeliac sprue exhibited the opposite finding of greater urinary isotope recovery than of luminal folate disappearance. Since the ileum is normally a poor site of absorption for PteGlu, these studies suggest functional hypertrophy—that is, adaptation—of folate absorption in the distal bowel of patients with coeliac sprue.

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