# The effect of dietary zinc deficiency on pancreatic γ-glutamyl hydrolase (*EC* 3.4.22.12) activity and on the absorption of pteroylpolyglutamate in rats

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**The effect of dietary zinc deficiency on γ-glutamyl hydrolase (*EC* 3.4.22.12) activity and on pteroylpolyglutamate absorption was investigated in rats. Enzyme activity was determined in pancreas and gut lumen washings. Pteroylpolyglutamate absorption was studied by determining the rise in plasma folate levels following pteroylpolyglutamate ingestion. Two experiments were performed; in each purified diets were given to three groups of immature male Wistar rats for approximately 2 weeks. One group was given a Zn-deficient diet *ad lib.* (ZD), the second was pair-fed daily with this group on a Zn-adequate diet (PF) and the third was given the Zn-adequate diet *ad lib.* (AL). In Expt 1, significantly reduced pancreatic γ-glutamyl hydrolase activity was observed in ZD rats. In Expt 2, pteroylpolyglutamate was administered on day 14 and in the 3 h period following pteroylpolyglutamate ingestion, lumen γ-glutamyl hydrolase activity and plasma folate levels were significantly lower in ZD rats. Pancreas is reported as the source of lumen γ-glutamyl hydrolase in rats. The results presented indicate that the pancreatic enzyme is Zn-sensitive. It was concluded that, as a result, γ-glutamyl hydrolase activity was reduced in the lumen of ZD rats. Consequently the hydrolysis and subsequent absorption of pteroylpolyglutamate was impaired in ZD rats, as indicated by the smaller rise in plasma folate levels that occurred following pteroylpolyglutamate ingestion. Results of this study concur with previous observations in human beings and rats that Zn deficiency has an adverse effect on folate metabolism.**

**Folate metabolism: Zinc: Rat.**

Dietary folates exist predominantly as a mixture of pteroylpolyglutamates (Butterworth *et al.* 1963) based on the 4-[(pteridin-6-ylmethyl)amino] benzoic acid skeleton conjugated with one or more L-glutamate units (Blakley, 1987). Intestinal absorption requires hydrolysis of the poly-γ-glutamyl side-chain to the absorbable mono- or diglutamyl derivative (Butterworth *et al.* 1969; Baugh *et al.* 1971). Hydrolysis is mediated by γ-glutamyl hydrolase (*EC* 3.4.22.12), commonly known as folate conjugase (Halsted, 1979).

Several studies have suggested an interaction between zinc and folate metabolism. Silink *et al.* (1975) observed that Zn is essential for the stability of purified bovine liver γ-glutamyl hydrolase. Human jejunal brush-border membrane γ-glutamyl hydrolase is activated by Zn (Dwivedi *et al.* 1983; Halsted *et al.* 1983, 1985; Wang *et al.* 1985; Chandler *et al.* 1986), and Zn was observed to stimulate activity in the supernatant fraction of rat intestinal mucosa (Day & Gregory, 1984). The latter finding was not confirmed by Wang *et al.* (1985). Several studies in human beings indicate that folate supplements adversely affect Zn metabolism (Milne *et al.* 1984; Lukaski *et al.* 1986; Sandstead *et al.* 1987; Simmer *et al.* 1987). In the rat, Wilson *et al.* (1983) suggested that folate competitively inhibits Zn absorption, and Ghishan *et al.* (1986) reported mutual inhibition between Zn and folate at the site of intestinal transport. Other studies do not indicate an adverse effect of folate on Zn metabolism in human beings (Wada *et al.* 1986; Keating *et al.* 1987; Butterworth *et al.* 1988) and in rats (Wada *et al.* 1986; Keating *et al.* 1987; Fuller *et al.* 1988). A number of

studies have suggested that Zn deficiency affects folate metabolism. Reduced liver (Williams *et al.* 1973) and plasma (Tamura *et al.* 1987) folate concentrations have been observed in Zn-deficient rats. Fuller *et al.* (1988) observed that Zn supplementation enhanced blood folate levels in rats. Impaired absorption of pteroylpolyglutamates, but not monoglutamates, was reported in Zn-depleted human beings (Tamura *et al.* 1978) and in aged rats (Kesavan & Noronha, 1983). Tamura *et al.* (1978) suggested that in man, intestinal γ-glutamyl hydrolase is a Zn-dependent enzyme, and that during Zn depletion its activity is decreased, resulting in reduced pteroylpolyglutamate absorption. Two separate intestinal γ-glutamyl hydrolases have been identified in man, one intracellular and the other located on the brush-border membrane (Reisenauer *et al.* 1977). The latter has been characterized as a Zn-activated exopeptidase and Chandler *et al.* (1986) reported that it, rather than the intracellular enzyme, is essential for the hydrolysis of dietary folates. Kesavan & Noronha (1983) observed decreased γ-glutamyl hydrolase activity in the pancreas and gut lumen of aged rats. They suggested that, in rats, pancreas is the source of lumen γ-glutamyl hydrolase.

In the present study γ-glutamyl hydrolase activity in the pancreas and gut lumen of Zn-deficient rats was investigated. The effect of Zn deficiency on pteroylpolyglutamate absorption was also investigated by determining the increase in plasma folate levels following ingestion of a yeast pteroylpolyglutamate preparation.

## MATERIALS AND METHODS

### *Animals and diets*

Two animal experiments were performed, involving twenty-seven rats in Expt 1 and ninety rats in Expt 2. Male weanling Wistar rats were obtained from the Biological Services Unit, University College, Cork, and maintained on commercial chow diet (balanced ration; William Connolly & Sons Ltd, Red Mills, Goresbridge, Co. Kilkenny) for approximately 1 week after delivery to our laboratory. Rats in each experiment were randomly allocated into three groups of similar mean weight. Each group was placed on one of three dietary regimens. The first group was fed on a Zn-deficient diet *ad lib.* (Zn-deficient; ZD), and the second group was fed *ad lib.* on a Zn-adequate diet (*ad lib.*-fed controls; AL). The third group was fed on the Zn-adequate diet, but each rat received daily the same amount of diet as consumed by a rat in the Zn-deficient group on the previous day (pair-fed controls; PF). Diets were formulated as shown in Table 1. The duration of Expt 1 was 16 d and the concentration of Zn in deficient and control diets was determined by analysis as 3·0 and 77·0 mg/kg diet respectively. The duration of Expt 2 was 14 d and on analysis, the Zn contents of the diets were 2·5 and 90·0 mg/kg in deficient and control diets respectively. All rats received distilled water *ad lib.*, daily food intake was recorded and animals were weighed every 4–5 d. Rats were kept in individual plastic cages fitted with stainless-steel bottoms and lids. Before each experiment, cages, hoppers and glass water bottles (fitted with melamine caps) were thoroughly detergent-washed and rinsed in distilled water to minimize Zn contamination. The room in which rats were housed was maintained at $22 \pm 2°$; a continuous air-flow system and a 12 h light–12 h dark cycle were operated.

On day 16, following overnight fasting, the rats in Expt 1 were lightly anaesthetized and blood was withdrawn by retro-orbital puncture of the subretinal vein, using heparinized capillary tubes. Plasma was prepared by centrifugation and portions stored in new plastic tubes at $-20°$. Rats were killed by cervical dislocation, the pancreas was removed, frozen in liquid nitrogen and stored at $-20°$ for later measurement of pancreatic γ-glutamyl hydrolase activity. The right tibia of each rat was excised and stored at $-20°$ for later measurement of Zn concentration.

Table 1. *Composition of diets (g/kg)*

| Diet ingredients | |
|---|---|
| Bipro* | 250 |
| Sucrose | 500 |
| Dextrose | 65 |
| Mineral mixture† | 35 |
| Vitamin mixture‡ | 50 |
| Maize oil§ | 100 |

* Bipro is a whey protein manufactured by Mitchelstown Creameries, Co. Cork, Irish Republic, containing about 13 mg Zn/kg and 920 g protein of biological value similar to that of egg protein/kg.

† Mineral mixture (American Institute of Nutrition, 1977) without zinc carbonate. Composition of mix (g/kg): calcium hydrogen phosphate 500, sodium chloride 74, potassium citrate monohydrate 220, potassium sulphate 52, magnesium oxide 24, manganous carbonate 3·5, ferric citrate 6, potassium iodate 0·01, sodium selenite 0·01, chromium potassium sulphate 0·55, cupric carbonate 0·3. Sucrose was added to 1 kg. Zinc was added as the carbonate to the required level for each control diet.

‡ Vitamin mixture, recommended by Queen Elizabeth College, London, and modified by D. A. Bender (personal communication). Composition of mix (g/kg): ascorbic acid 3·75, nicotinic acid 3, vitamin $B_{12}$ (0·1 %) 0·04, calcium pantothenate 2, thiamin hydrochloride 0·5, riboflavin 5′-phosphate 0·5, pyridoxine hydrochloride 0·5, pteroylmonoglutamic acid 0·2, biotin 0·5, menadione 0·5, Rovimix $A/D_3$ (150 mg vitamin A and 2·5 mg vitamin D/g) 0·4, Rovimix E (500 mg/g) 7·5, choline chloride 90, cellulose 891.

§ Mazola® Pure Corn Oil; CPC Ltd, Esher, Surrey.

On days 11, 12 and 13, rats in Expt 2 were administered intraperitoneally 80 $\mu$g pteroylmonoglutamic acid/kg body-weight in order to minimize variations in initial folate status. On day 14, following overnight fasting, each group of thirty rats was divided into five subgroups of six. Rats were administered intragastrically 40 $\mu$g yeast pteroylpolyglutamate/kg body-weight. Pteroylpolyglutamate was prepared from yeast, as described by Perry & Chanarin (1968) and found to contain 102 (SE 6·0) mg folate/kg. One subgroup from each group was bled at 0, 0·5, 1, 2 and 3 h after pteroylpolyglutamate ingestion and plasma was prepared as described previously. Small intestines were removed and divided into four segments of equal length. The lumen contents of the proximal segment were washed out with 10 ml ice-cold 0·25 M-sucrose solution and stored at −20°.

*Analytical procedures*

Zn concentrations in diets, plasma and tibia were determined by flame atomic absorption spectroscopy (Pye Unicam SP9 Model). Diets and tibia were digested in a mixture (2:1; v/v) of Analar grade concentrated nitric and perchloric acids (BDH Chemicals Ltd, Poole, Dorset) on a hot plate until the colour resembled that of the reagent blanks. Samples were made to volume (variable) with distilled water. Aqueous Zn standards were prepared by dilution of Zn nitrate stock solution (Spectrosol; BDH Chemicals Ltd). Before digestion tibia were cleansed of soft tissue and dried overnight at 105° to a constant weight. Plasma was diluted with distilled water (1:4) and Zn standards were prepared in glycerol (50 ml/l).

Plasma folate concentrations were measured microbiologically by the method of Scott *et al.* (1974) as modified by Wilson & Horne (1982) using *Lactobacillus casei* (ATCC 7469). Pancreatic $\gamma$-glutamyl hydrolase activity was determined by a modification of the method of Kesavan & Noronha (1983). An homogenate of pancreas (100 mg/ml) was prepared in ice-cold 0·25 M-sucrose using a Potter–Elvehjem glass tube and Teflon pestle. The homogenates were centrifuged at 8600 ***g*** for 15 min at 4° using a Sorvall RC-5B centrifuge. The supernatant fraction was removed by micropipette, portions transferred to new plastic tubes and stored at −20° until required. To determine pancreatic $\gamma$-glutamyl hydrolase

Table 2. *Expts 1 and 2. Body-weight changes, food intake and food conversion efficiency values of zinc-deficient (ZD) rats and their pair-fed (PF) and* ad lib.*-fed (AL) controls*

(Mean values and standard deviations)

| | Expt 1 | | | | | Expt 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Statistical significance of difference from ZD group: *P* | | | | | Statistical significance of difference from ZD group: *P* | |
| Dietary group*... | ZD | PF | AL | PF | AL | ZD | PF | AL | PF | AL |
| *n*... | 9 | 9 | 9 | | | 30 | 30 | 30 | | |
| Initial wt (g) | | | | | | | | | | |
| Mean | 133·4 | 134·7 | 136·0 | NS | NS | 122·1 | 122·1 | 124·5 | NS | NS |
| SD | 11·3 | 10·7 | 8·0 | | | 5·7 | 6·1 | 9·2 | | |
| Final wt (g) | | | | | | | | | | |
| Mean | 155·6 | 173·4 | 201·6 | < 0·05 | < 0·001 | 143·6 | 150·1 | 176·8 | NS | < 0·001 |
| SD | 14·7 | 14·4 | 7·7 | | | 11·5 | 9·5 | 12·4 | | |
| Wt gain (g) | | | | | | | | | | |
| Mean | 20·9 | 38·7 | 65·1 | < 0·001 | < 0·001 | 21·3 | 28·4 | 52·0 | < 0·05 | < 0·001 |
| SD | 6·4 | 8·9 | 10·0 | | | 8·7 | 10·6 | 8·5 | | |
| Food intake (g) | | | | | | | | | | |
| Mean | 183·4 | 181·6 | 256·8 | NS | < 0·001 | 177·1 | 175·9 | 228·4 | NS | < 0·001 |
| SD | 20·9 | 18·3 | 13·1 | | | 15·7 | 13·2 | 13·3 | | |
| Food conversion efficiency† | | | | | | | | | | |
| Mean | 0·12 | 0·21 | 0·26 | < 0·001 | < 0·001 | 0·12 | 0·16 | 0·23 | < 0·02 | < 0·001 |
| SD | 0·03 | 0·04 | 0·03 | | | 0·04 | 0·05 | 0·03 | | |

NS, not significant.
* For details of diets, see p. 186 and Table 1.
† g body-weight gain/g food eaten.

activity the following reagents were added to new glass tubes: 0·7 ml 0·1 M-acetate buffer, pH 4·1, ascorbate (10 g/l), 0·1 ml $\beta$-mercaptoethanol (100 ml/l buffer) and 0·2 ml yeast extract (300 mg made to 100 ml with buffer). The reaction was initiated by adding 5–10 $\mu$l pancreas supernatant fraction. The mixture was incubated in a water-bath at 37° for 20 min and the reaction terminated by heating in a boiling water-bath for 10 min. Blanks were prepared by heating the mixture (excluding yeast extract) in a boiling water-bath for 10 min, cooling, adding the yeast extract and incubating at 37° for 20 min. Samples were later analysed microbiologically for free folate. Enzyme activity was expressed as ng folate released/mg protein per 20 min. Lumen $\gamma$-glutamyl hydrolase was similarly determined. The protein concentration of homogenates and lumen solutions was determined by the method of Lowry *et al.* (1951).

### *Statistical analysis*

Means, standard deviations and the significance values (*P*) were calculated using the Minitab Statistical Package (Ryan *et al.* 1976) in a Vax computer (Model 11/780).

## RESULTS

In both experiments anorexia and a cyclic eating pattern were observed in rats fed on the Zn-deficient diet for 3–4 d. Consequently, food intake was significantly ($P < 0·001$)

Table 3. *Expt 1. The effect of zinc deficiency on plasma (mg/l) and tibia (μg/g) Zn concentrations* and pancreatic γ-glutamyl hydrolase (folate conjugase, EC 3.4.22.12) activity in Zn-deficient (ZD) rats and their pair-fed (PF) and* ad-lib.*-fed (AL) controls*

(Mean values and standard deviations)

| Dietary group†... | | ZD | PF | AL | Statistical significance of difference from ZD group: *P* PF | AL |
|---|---|---|---|---|---|---|
| *n*... | | 9 | 9 | 9 | | |
| Plasma Zn (mg/l) | | | | | | |
| | Mean | 1·27 | 1·81 | 1·80 | < 0·001 | < 0·001 |
| | SD | 0·09 | 0·14 | 0·21 | | |
| Tibia Zn (μg/g) | | | | | | |
| | Mean | 115·9 | 248·8 | 261·5 | < 0·001 | < 0·001 |
| | SD | 10·3 | 21·9 | 11·5 | | |
| γ-Glutamyl hydrolase activity (ng folate per mg protein/20 min) | | | | | | |
| | Mean | 330·0 | 790·0 | 830·0 | < 0·001 | < 0·001 |
| | SD | 30·0 | 50·0 | 100·0 | | |

* Concentrations (/g dry weight).
† For details of diets, see p. 186 and Table 1.

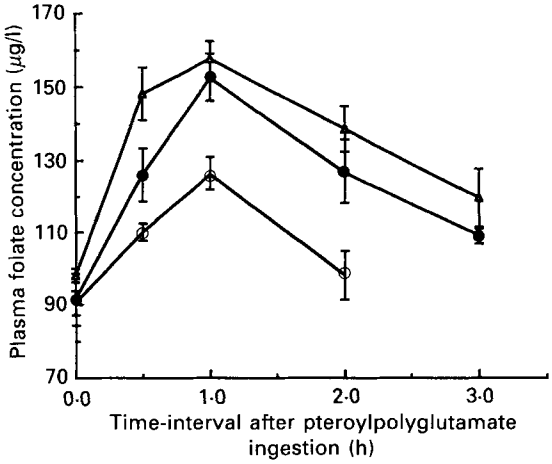


Fig. 1. Expt 2. Plasma folate concentrations (μg/l) in rats after intragastric administration of yeast pteroylpolyglutamate. Points are means, and standard errors represented by vertical bars, for: (○), Zn-deficient; (●), pair fed controls; (△), *ad-lib.*-fed controls; for details of diets, see p. 186 and Table 1.

reduced for the ZD and PF groups compared with the AL (control) group (Table 2). Significant reductions in food conversion efficiency ($P < 0{\cdot}001$; $P < 0{\cdot}02$) and weight gain ($P < 0{\cdot}001$; $P < 0{\cdot}05$) were observed in ZD groups compared with PF groups in Expts 1 and 2 respectively (Table 2).

Table 3 shows the effect of Zn deficiency on tissue Zn levels and pancreatic γ-glutamyl hydrolase activity in Expt 1. Plasma and tibia Zn concentrations were significantly ($P <$

Table 4. *γ-Glutamyl hydrolase (folate conjugase, EC 3.4.22.12) activity* in lumen washings of the proximal intestinal segment of zinc-deficient (ZD) rats and their pair-fed controls (PF) after ingestion of pteroylpolyglutamate*

(Mean values and standard deviations)

| Dietary group†... | | ZD | PF | |
|---|---|---|---|---|
| *n*... | | 6 | 6 | |
| Time-interval after ingestion of pteroylpolyglutamate (h) | | | | Statistical significance of difference between PF and ZD groups: *P* |
| 0 | Mean | 78·5 | 135·3 | < 0·001 |
| | SD | 14·8 | 25·0 | |
| 0·5 | Mean | 152·8 | 194·3 | < 0·05 |
| | SD | 26·7 | 27·2 | |
| 1·0 | Mean | 112·4 | 190·0 | < 0·001 |
| | SD | 30·4 | 21·0 | |
| 2·0 | Mean | 74·6 | 171·5 | < 0·001 |
| | SD | 18·9 | 21·0 | |
| 3·0 | Mean | — | 133·9 | |
| | SD | — | 23·6 | |

* ng folate/mg protein per 20 min.
† For details of diets, see p. 186 and Table 1.

0·001) lower in the ZD group than in the PF and AL controls. No difference was observed in plasma and tibia Zn levels in the latter groups, indicating that the reduction observed in the ZD group was not due to decreased food intake. Similarly, pancreatic γ-glutamyl hydrolase activity was significantly ($P < 0·001$) lower in the ZD group than in the PF and AL controls. The results in Fig. 1 are those of the in vivo pteroylpolyglutamate absorption study performed in Expt 2. Baseline plasma folate concentration and changes in plasma folate concentration that occurred at various time-intervals following pteroylpolyglutamate ingestion are indicated. Following ingestion, plasma folate concentration was significantly lower in the ZD group compared with the AL control group at 0·5, 1, and 2 h ($P < 0·001$) and with the PF control group at 1 and 2 h ($P < 0·01$ and $P < 0·03$ respectively). At 2 h post-ingestion, plasma folate concentration for the ZD group had returned to baseline values. The area under the folate absorption curve for the ZD group represented 26 and 38% of that obtained for the AL and PF control groups respectively. Table 4 shows the γ-glutamyl hydrolase activity of the gut lumen washings. The fasting level of gut lumen γ-glutamyl hydrolase activity in the ZD group was approximately 50% lower than that for the PF group. Lumen γ-glutamyl hydrolase activity increased in both groups following pteroylpolyglutamate ingestion, attaining maximal levels within 1 h. Thereafter, activity progressively declined, returning to fasting levels at 2 and 3 h post-ingestion in the ZD and PF groups respectively.

## DISCUSSION

In rats, several authors have reported γ-glutamyl hydrolase activity in the intracellular supernatant fraction of intestinal mucosal homogenates (Day & Gregory, 1984; Elsenhans *et al.* 1984; Wang *et al.* 1985). Activity in the brush-border membrane is reported as negligible (Wang *et al.* 1985). Following oral administration of pteroylpolyglutamate to rats, Jagerstad & Westesson (1974) observed partially hydrolysed derivatives in the

stomach and duodenal contents, but the absorbable derivative occurred mainly beyond the point of entry of the pancreatic duct. Consequently, they suggested that γ-glutamyl hydrolase is secreted from the pancreas into the intestine. Similarly, Kesavan & Noronha (1983) suggested that lumen γ-glutamyl hydrolase of pancreatic origin has a greater role in pteroylpolyglutamate digestion than the mucosal enzyme.

The results of Expt 1 indicate that rat pancreatic γ-glutamyl hydrolase is a Zn-dependent enzyme. In ZD rats enzyme activity was 60% lower than in PF and AL controls. The occurrence of Zn deficiency was indicated by significantly lower plasma and tibia Zn concentrations in the ZD group compared with both PF and AL control groups (Table 3). Impaired food utilization and a cyclic eating pattern, characteristics of Zn deficiency in rats (Williams & Mills, 1970), were also observed. Reduced enzyme activity was not due to decreased food intake, as similar levels of activity were observed in both PF and AL control rats (Table 3).

The results of Expt 2 confirm the observation of Tamura *et al.* (1978) that dietary Zn deprivation adversely affects pteroylpolyglutamate absorption. The protocol for Expt 1 was repeated to ensure the development of a similar state of Zn deficiency. At zero-time all groups exhibited similar plasma folate concentrations, since all were supplemented with pteroylmonoglutamic acid for 3 d before the absorption study. Compared with PF and AL control groups, pteroylpolyglutamate absorption was impaired in the ZD group, as indicated by the reduced area under the plasma folate absorption curve. Likewise, significantly lower plasma folate concentration was determined at various time-intervals following pteroylpolyglutamate ingestion in the ZD group compared with the PF and AL groups (Fig. 1). The results of Expt 2 suggest that lumen γ-glutamyl hydrolase is also a Zn-sensitive enzyme. In the lumen, γ-glutamyl hydrolase activity was significantly lower at zero-time and less activity was induced following pteroylpolyglutamate ingestion in the ZD group compared with the pair-fed control group (Table 4). The results of both experiments demonstrate that pancreatic and lumen γ-glutamyl hydrolases are highly sensitive to the Zn status of rats, the activity of both being 50–60% lower in Zn-deficient compared with control rats. Kesavan & Noronha (1983) observed that, in rats, both pancreatic and lumen γ-glutamyl hydrolases have similar pH optima and that the activity of both increases following pteroylpolyglutamate ingestion. The finding that both enzymes are equally sensitive to dietary Zn deprivation supports the observations of Jagerstad & Westesson (1974) and Kesavan & Noronha (1983) that pancreas is the source of the lumen enzyme in the rat. The latter authors also observed that following pteroylpolyglutamate ingestion, peak lumen γ-glutamyl hydrolase activity occurred concurrently with the peak rise in plasma folate concentration. Similar results were obtained in the present study (Fig. 1; Table 4), indicating that lumen γ-glutamyl hydrolase is active in the hydrolysis of pteroylpolyglutamate.

The present results suggest that as a consequence of reduced γ-glutamyl hydrolase activity in Zn-deficient rats, pteroylpolyglutamate hydrolysis is impaired. This is indicated by the smaller increase in plasma folate concentration that occurred following ingestion of pteroylpolyglutamate in ZD rats compared with PF and AL control rats. Similarly, Chandler *et al.* (1986) suggested that, in human beings, brush-border γ-glutamyl hydrolase activity and dietary folate availability are influenced by intestinal Zn levels. However, absorption rather than hydrolysis is considered the rate-limiting step in folate metabolism in man (Reisenauer & Halsted, 1987) and in rats (Dhar *et al.* 1977; Rosenberg & Selhub, 1986). Hydrolysis is reported as rate-limiting in aged rats (Kesavan & Noronha, 1983) and may be rate-limiting in Zn-deficient rats. Zn may also have a role in the transfer of absorbable folate across the gut wall. In rats receiving the vitamin in the monoglutamyl form, Zn supplementation enhanced blood folate levels (Fuller *et al.* 1988). Therefore, it is

possible that Zn deficiency reduces the rates of both pteroylpolyglutamate hydrolysis and pteroylmonoglutamate absorption. Increased investigation of the role of Zn in the hydrolysis and absorption of dietary folates is required to elucidate further the effect of Zn deficiency on folate metabolism.

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