The effect of dietary zinc depletion and repletion on rats: Zn concentration in various tissues and activity of pancreatic γ-glutamyl hydrolase (*EC* 3.4.22.12) as indices of Zn status

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Unlike severe zinc deficiency, marginal Zn deficiency is difficult to identify in rats because no reliable indicator of suboptimal Zn status is currently available. We have previously observed reduced pancreatic γ-glutamyl hydrolase (*EC* 3.4.22.12) activity and impaired pteroylpolyglutamate absorption in Zn-deficient rats. In the present study the effect of Zn depletion and repletion on the Zn concentration of various tissues and on the activity of this enzyme was investigated. The objective was to determine the sensitivity of these variables to Zn depletion and to evaluate their usefulness as indices of Zn status. Male Wistar rats (about 180 g), maintained from weanling on a purified Zn-adequate diet, were randomly allocated into twelve groups. A pretreatment control group was killed immediately. The remaining eleven groups were fed on a Zn-deficient diet and a group killed daily for 7 d (Zn-depleted groups). The remaining four groups were re-fed the Zn-adequate diet and a group killed daily (Zn-repleted groups). On analysis, pancreas and spleen Zn levels responded most rapidly to reduced Zn intake, followed by tibia, liver, kidney and plasma. Zn concentration was maintained in testes. Reduced plasma folate levels were also observed. A significant reduction in pancreatic γ-glutamyl hydrolase activity before the depletion of many tissue Zn stores confirms the Zn sensitivity of the enzyme. It was concluded that future investigation into the inter-relationship between Zn and folate metabolism may be useful in identifying a sensitive, biochemical index of Zn status.

Folate: γ-Glutamyl hydrolase: Zinc status: Rat

It is generally accepted that, with the notable exception of iron, methods for the assessment of trace element status in humans remain grossly inadequate (Hambidge, 1988). Clinical diagnosis of severe zinc deficiency in man is relatively simple because of the development of a characteristic skin rash. Severe Zn deficiency in growing animals is characterized by anorexia, skeletal abnormalities, skin lesions, alopecia and inhibition of sexual maturity (Burch *et al.* 1975). Assessment of subclinical Zn deficiency is more difficult and no reliable indicator of suboptimal Zn intake by either man (King, 1986; Hambidge, 1988) or animals (Mathur *et al.* 1978; Mills, 1987) is currently available.

In an earlier study (Canton *et al.* 1989), reduced γ-glutamyl hydrolase (*EC* 3.4.22.12) activity in pancreas and gut lumen and impaired pteroylpolyglutamate absorption were observed in Zn-deficient rats. Naturally occurring folates exist predominantly as pteroylpolyglutamates (Butterworth *et al.* 1963) and γ-glutamyl hydrolase is required for their digestion in man (Butterworth *et al.* 1969) and rats (Hepner, 1969). In rats, pteroylpolyglutamates are hydrolysed in the intestinal lumen to monoglutamyl folate before tissue uptake of the latter (Darcy-Vrillon *et al.* 1988). Hydrolysis is reported to be mediated by lumen γ-glutamyl hydrolase of pancreatic origin (Jagerstad & Westesson, 1974; Kesavan & Noronha, 1983). Dietary folates are hydrolysed in humans by brush-border membrane γ-glutamyl hydrolase, which is a Zn-activated enzyme (Chandler *et al.* 1986). The results of our earlier study indicated that rat pancreatic γ-glutamyl hydrolase

is also a Zn-sensitive enzyme and that pteroylpolyglutamate hydrolysis is impaired in the gut lumen of Zn-deficient rats leading to reduced pteroylpolyglutamate absorption (Canton *et al.* (1989).

In the present study we investigated the effect of short-term Zn depletion and repletion on the concentration of Zn in various tissues of the rat and on the activity of pancreatic γ-glutamyl hydrolase. Plasma folate levels were also determined. The objective was to determine the sensitivity of the enzyme to dietary Zn depletion and to evaluate its usefulness as a reliable indicator of suboptimal Zn status in rats.

MATERIALS AND METHODS

Animals and diets

Rats used in the present study were obtained from the same source, similarly acclimatized, maintained under identical conditions and fed on the same whey protein-based diets as previously described (Canton *et al.* 1989).

Following acclimatization, sixty male Wistar rats were fed on a Zn-adequate control diet until they weighed approximately 180 g. On analysis, the diet contained 80·0 mg Zn/kg diet. Rats were then randomized into twelve groups of five of approximately equal weight. One group was starved overnight and killed the following day. This constituted the pretreatment control group. The remaining eleven groups were fed on a Zn-deficient diet *ad lib*. On analysis this diet contained 2·5 mg Zn/kg diet. Following overnight starvation a group of rats was killed daily for the subsequent 7 d. These rats constituted the seven Zn-depleted groups (ZD 1–7). On day 8, the remaining four groups were re-fed the control Zn-adequate diet and following overnight starvation a group was killed daily for the next 4 d. These rats constituted the four Zn-repleted groups (ZR 1–4).

Collection of samples

Plasma was prepared and pancreas, spleen, liver, kidneys, testes and tibia were removed and stored at $-20°$, as previously described (Canton *et al.* 1989).

Analytical procedures

Zn in diets and tissues was determined by atomic absorption spectrophotometry (Pye-Unicam SP9 Model; Pye-Unicam Ltd, Cambridge), as previously described (Canton *et al.* 1989). Plasma folate concentrations were estimated microbiologically by the method of Scott *et al.* (1974), as modified by Wilson & Horne (1982) using *Lactobacillus casei* (ATCC 7469). Pancreatic γ-glutamyl hydrolase activity was determined in tissue homogenates using a modification of the method of Kesavan & Noronha (1983), which was previously described (Canton *et al.* 1989). Enzyme activity was expressed as ng folate released/mg protein per 20 min. The protein content of homogenates was determined by the method of Lowry *et al.* (1951).

Statistical analysis

In estimating the significance of differences between means for the experimental groups and the pretreatment control group, we assumed that each group had approximately the same variance, and carried out unpaired t tests.

RESULTS

The concentration of Zn in pancreas, spleen, tibia, liver, kidney and plasma decreased significantly during Zn depletion and increased during repletion (Figs 1(*b*)–3(*c*)), whereas the concentration of Zn in testes was unchanged throughout the 11 d regimen (Fig. 1(*a*)).

Pancreas and spleen exhibited the greatest change in Zn concentration (Figs 1(b), (c)). The sensitivity of pancreas to dietary Zn intake was indicated by the significant ($P < 0.05$; $P < 0.01$; $P < 0.001$) reductions observed in pancreatic Zn levels after giving a Zn-deficient diet for 2, 3 and 4 d respectively, and by the increase to pretreatment control levels when Zn was replaced in the diet for 1 d (Fig. 1(b)). The spleen was also sensitive to depletion; significantly lower Zn levels were observed in all groups fed on the Zn-deficient diet (Fig. 1(c)). A significant ($P < 0.001$) reduction in tibia Zn concentration was observed in rats fed on the Zn-deficient diet for 3 d. Tibia Zn levels increased gradually during repletion, but remained significantly ($P < 0.05$) lower than pretreatment controls until the Zn-adequate diet had been given for 4 d (Fig. 2(a)). Rats fed on the Zn-deficient diet for 3 d had significantly ($P < 0.05$) lower liver Zn levels than pretreatment controls; the maximum reduction occurring after 7 d when a 21 % reduction was observed (Fig. 2(b)). The kidney responded more slowly to depletion; only the ZD 6 and ZD 7 ($P < 0.05$) groups exhibited significant reductions in kidney Zn levels (Fig. 2(c)). Plasma Zn levels were also slow to respond to depletion; a significant ($P < 0.05$) reduction was observed only in the ZD 7 group. Plasma Zn levels increased in Zn-depleted rats; concentrations significantly ($P < 0.05$) greater than pretreatment controls were observed in the ZR 1 group (Fig. 3(a)). Plasma folate levels also responded to dietary Zn depletion. Significant ($P < 0.05$) reductions were observed in the ZD 6 and ZD 7 groups following a 30 % decrease in plasma folate concentration (Fig. 3(b)). Pancreatic γ-glutamyl hydrolase activity was significantly ($P < 0.001$) decreased in the ZD 3 group, activity being approximately 60 % lower than in the pretreatment control group. No further decline in activity was observed during the remaining days of Zn-deficient nutrition. Zn repletion resulted in an immediate increase in enzyme activity to control levels (Fig. 3(c)).

DISCUSSION

Findings from the present study identify pancreas, spleen, tibia, liver, kidney and plasma as tissues where the Zn concentration was responsive to short-term Zn depletion. The pancreas is reported to be highly sensitive to Zn depletion in rats (Williams & Mills, 1970; Kramer, 1984; Southon et al. 1988), sheep (Ott et al. 1964) and pigs (Crofton et al. 1983). In the present study the concentration of Zn in the spleen was also sensitive to depletion. This finding is in agreement with that of Keen et al. (1985), who observed reduced spleen Zn levels in rats fed on a Zn-deficient diet, but contrary to that of Giugliano & Millward (1984), who reported no effect. The reduction observed in tibia Zn levels confirms the long-held observation that bone is a Zn-sensitive tissue in rats (Hove et al. 1938). In this context, Momcilovic et al. (1975) suggested that femur Zn concentration accurately reflects the Zn status of weanling rats. However, as far as other tissues are concerned there are mixed findings in the literature concerning the effects of Zn-deficient diets. Prasad et al. (1967) and Taylor et al. (1988) observed that liver Zn concentration in rats was not affected by consumption of Zn-deficient diets. On the other hand, Mathur et al. (1978) and Keen et al. (1988) reported that liver Zn concentration reflected dietary Zn intake and could be used as an index of Zn status in rats and monkeys respectively. Results of the present study are in agreement with the latter observations. Keen et al. (1985) observed no change in kidney Zn levels in rats fed on low-Zn diets, whereas results of the present study and those of Giugliano & Millward (1984) indicated that levels of Zn in kidney were responsive to depletion. There are many reports of reduced testis Zn levels in severely Zn-restricted rats (Prasad et al. 1967; Giugliano & Millward, 1984; Law et al. 1988). However, results of the present study show that testis Zn concentration is not affected by a regimen of short-term Zn depletion.

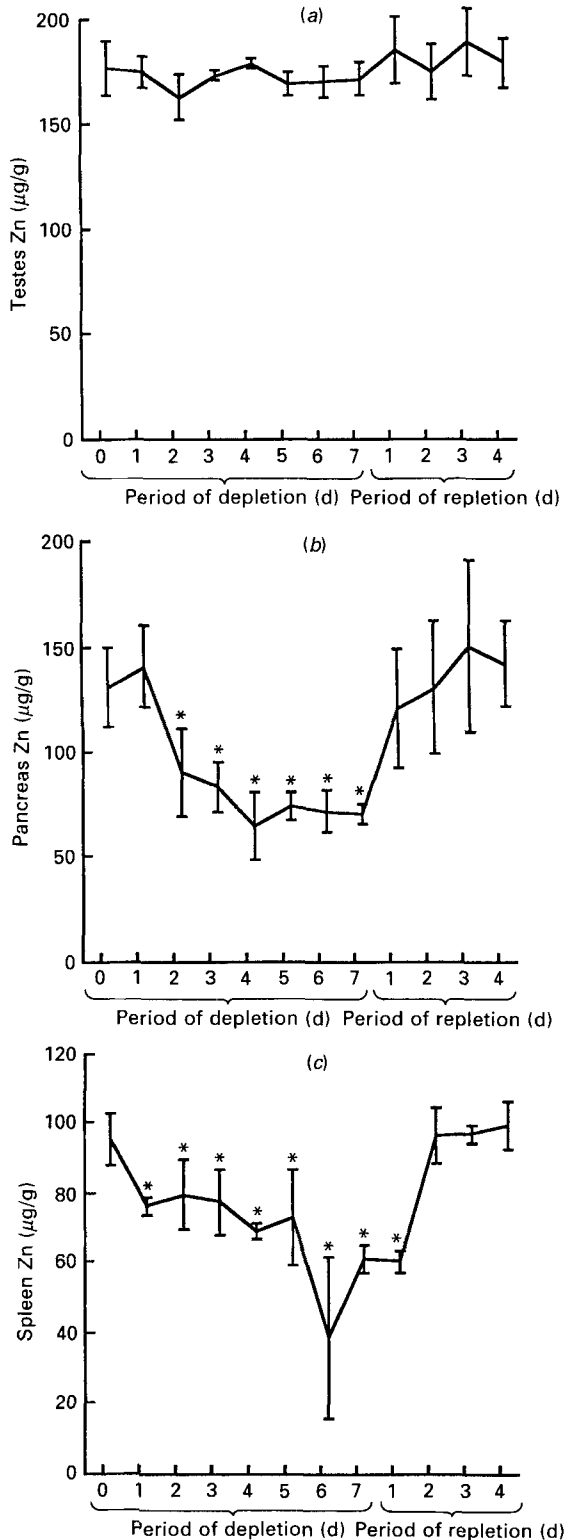Blood serum or plasma can be obtained with minimum interference with the animal, and

Fig. 1. Effect of zinc depletion and repletion on tissue Zn concentrations (μg/g dry weight) in (a) testes, (b) pancreas, (c) spleen. Values are means, and standard deviations are represented by vertical bars. Mean values were significantly different from those for the pretreatment control group: * $P < 0.05$.
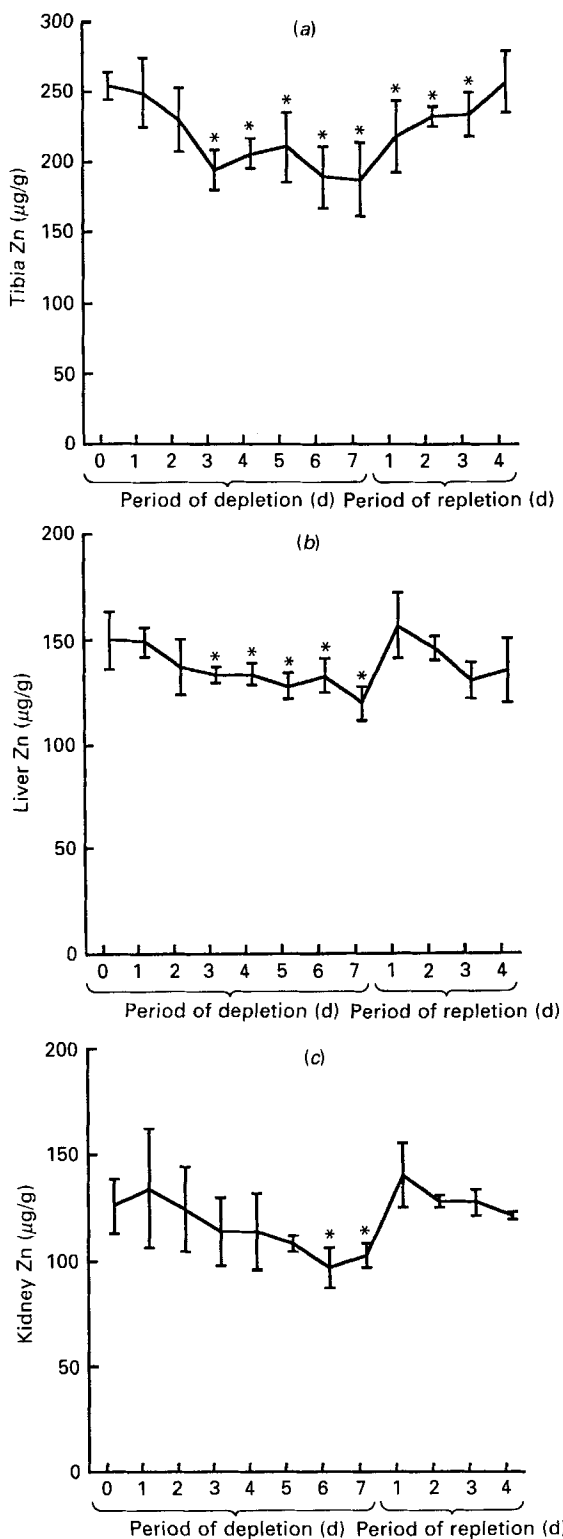
Fig. 2. Effect of zinc depletion and repletion on tissue Zn concentrations (μg/g dry weight) in (a) tibia, (b) liver, (c) kidney. Values are means, and standard deviations are represented by vertical bars. Mean values were significantly different from those for the pretreatment control group: * $P < 0.05$.
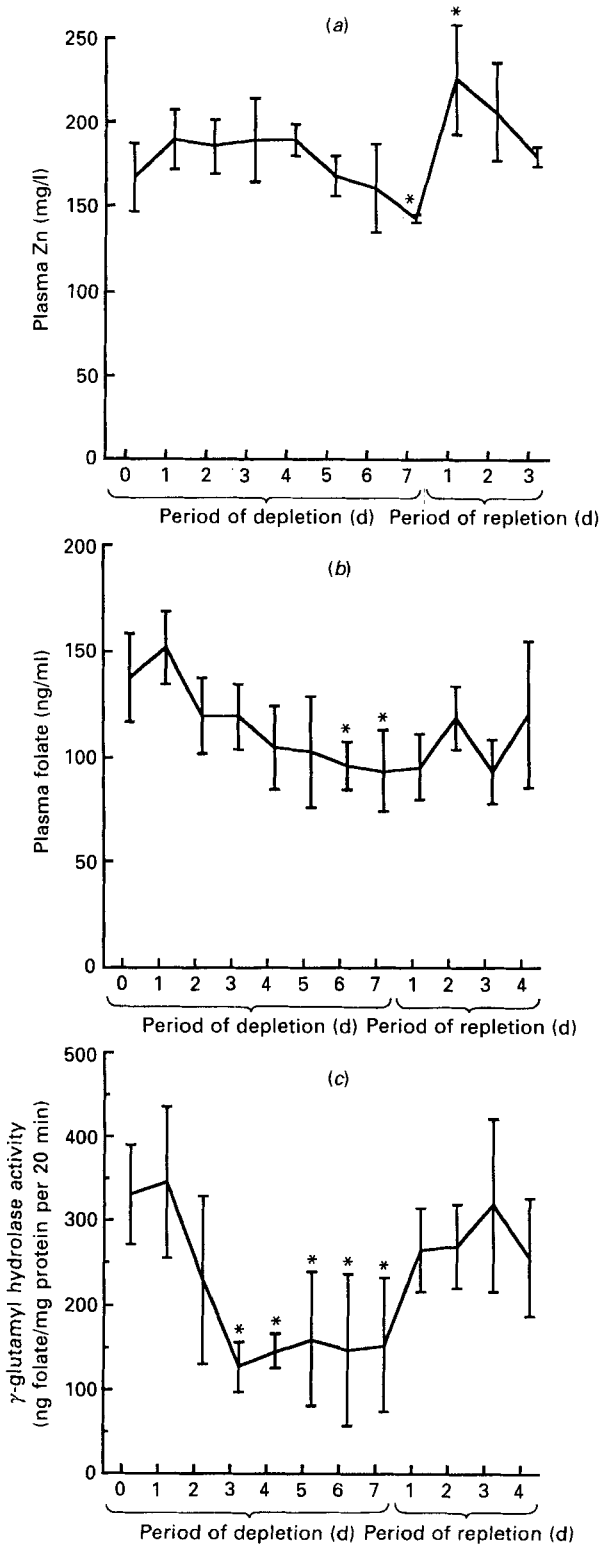
Fig. 3. Effect of zinc depletion and repletion on (a) plasma Zn concentration, (b) plasma folate concentration, (c) pancreatic γ-glutamyl hydrolase (EC 3.4.22.12) activity. Values are means, and standard deviations are represented by vertical bars. Mean values were different from those for the pretreatment control group: * $P <$ 0·05.

many researchers report reduced levels of circulating Zn in Zn-deficient rats (Wilkins *et al.* 1972; Brown *et al.* 1978; Kramer, 1984; Milne *et al.* 1985). However, plasma Zn is not considered a reliable indicator of Zn status because factors other than reduced Zn status cause hypozincaemia in rats (Chesters & Will, 1978; Sato *et al.* 1984). In the present study plasma Zn concentration was not significantly ($P < 0.05$) reduced until the Zn-deficient diet had been given for 7 d, indicating that plasma is not as sensitive to depletion as other tissues studied.

Due to limitations associated with determining tissue Zn levels as indices of Zn status in humans, the possibility of measuring the activity of Zn-dependent enzymes as an index of Zn status has been considered (Solomons, 1979). Results of a previous study indicated that in rats, pancreatic γ-glutamyl hydrolase is Zn-sensitive (Canton *et al.* 1989). In the present study a 60 % reduction in activity of this enzyme after 3 d of Zn-deficient nutrition confirms the earlier finding. The decrease in enzyme activity observed in the present study was concomitant with a 50 % reduction in pancreatic Zn levels. This finding is consistent with previous observations that the pancreas responds rapidly to a reduction in dietary Zn intake (Williams & Mills, 1970; Roth & Kirchgessner, 1981; Southon *et al.* 1988). Mills *et al.* (1967) reported a 48 % reduction in pancreatic carboxypeptidase (EC 3.4.2.1) activity in rats fed on a Zn-deficient diet for 4 d. Similarly, Roth & Kirchgessner (1980) cited an earlier study in which they observed a 25 % reduction in pancreatic carboxypeptidase activity in rats after 2 d of Zn depletion and a return to control levels within 3 d of repletion. Williams & Mills (1970) suggested that reduced activity of this enzyme was a consequence of reduced pancreatic Zn levels, less Zn being available for incorporation into the metalloenzyme. Results of the current study indicate that a similar relationship may exist between pancreatic Zn levels and pancreatic γ-glutamyl hydrolase activity. The observation that both variables were maximally reduced after 4 d of Zn depletion and that no further decline occurred during the next 3 d supports the hypothesis that the reduction in enzyme activity is attributable to reduced Zn levels in pancreas. That a significant reduction in the activity of this enzyme was detectable before the depletion of many tissue stores is of interest regarding its utilization as an index of Zn status.

We have previously reported an inter-relationship between Zn status and folate metabolism based on the observations of reduced pancreatic and gut lumen γ-glutamyl hydrolase activity and reduced pteroylpolyglutamate absorption in Zn-deficient rats (Canton *et al.* 1989). In man, Chandler *et al.* (1986) suggested that the reduction in pteroylheptaglutamate absorption, as observed by Tamura *et al.* (1978) in Zn-depleted subjects, was a consequence of reduced activity of the Zn-activated brush-border membrane γ-glutamyl hydrolase. Our earlier observations led us to suggest that, due to the Zn sensitivity of pancreatic γ-glutamyl hydrolase, a similar relationship existed in rats. However, results obtained in the current study indicate that Zn may also have a role in the transfer of folic acid across the gut wall. In rats, folate transport rather than hydrolysis is considered the rate-limiting step in metabolism (Darcy-Vrillon *et al.* 1988). Rats in the present study received dietary folate in the monoglutamyl form, consequently the reduction in plasma folate levels observed indicates that dietary Zn may also influence folate transport. In this context, Tamura *et al.* (1987) observed reduced plasma folate levels in Zn-deficient rats, and Fuller *et al.* (1988) observed that Zn supplementation enhanced blood folate levels in rats.

In summary, the results presented confirm earlier observations of the sensitivity of pancreatic γ-glutamyl hydrolase to Zn in rats and also indicate that the activity of this enzyme is more sensitive to dietary Zn depletion than the concentration of Zn determined in various tissues. In addition, the observed reduction in plasma folate levels suggests that the inter-relationship between Zn status and folate metabolism in rats may encompass both pteroylpolyglutamate hydrolysis and pteroylmonoglutamate absorption.

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