

Analysis of cyclic feed intake in rats fed on a zinc-deficient diet and the level of dihydropyrimidinase (*EC* 3.5.2.2)

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The body weight and feed intake of rats fed on a Zn-deficient diet for 28 d were reduced compared with those of control rats. The feed intakes of the Zn-deficient and control groups during the period were 10.2 (SE 0.3) and 15.7 (SE 0.2) g/d respectively. Cyclic variations in feed intake and body-weight changes were found in analysis not only of all the data for five rats but also that in each individual rat. Cosinor analysis revealed that the cyclical period of both the feed intake and body-weight change in the Zn-deficient rats was 3.5 (SE 0.1) d. The mesor and amplitude value of the feed intake in the Zn-deficient rats was 10.1 (SE 0.4) g/d and 3.5 (SE 0.5) g/d respectively, and that of body-weight change was 1.4 (SE 0.1) g/d and 7.9 (SE 1.3) g/d respectively. Among pyrimidine-catabolizing enzymes, dihydropyrimidinase (*EC* 3.5.2.2) activity showed significant retardation in the Zn-deficient rat liver with decrease of the enzyme protein. The ratio of apo-form to holo-form dihydropyrimidinase in the liver was not affected by the Zn-deficient diet.

Zinc deficiency: Cosinor analysis: Dihydropyrimidinase

It is well recognized that Zn is essential for growth in animals (Williams & Mills, 1970) as well as man (Golden, 1988). Failure of growth during Zn deficiency *in vivo* is complex, with cyclical changes in feed intake (Mills *et al.* 1969; Chesters & Quarterman, 1970; Chesters & Will, 1973; Wallwork *et al.* 1981; Gingliano & Millward, 1984; Kramer *et al.* 1984; Quinn *et al.* 1990), body weight (Wallwork *et al.* 1981; Gingliano & Millward, 1984), and muscle mass (Gingliano & Millward, 1984). Zn deficiency impairs growth by a combination of decreased feed intake, lowered anabolic response to the feed, and increased catabolic response (Gingliano & Millward, 1984). Recently, it was proposed that metabolic hormones such as insulin, somatotropin, insulin-like growth factor, and corticosterone may be mediating factors in the impaired growth (Gingliano & Millward, 1987; Droke *et al.* 1993).

Dihydropyrimidinase (5,6-dihydropyrimidine amidohydrolase, *EC* 3.5.2.2) is the second enzyme in pyrimidine catabolism. Mammalian dihydropyrimidinase was originally studied (Wallach & Grisolia, 1957), purified and characterized in calves (Maguire & Dudley, 1978; Kautz & Schnackerz, 1989), cows (Brooks *et al.* 1983; Lee *et al.* 1986, 1987), and rats (Maguire & Dudley, 1978; Kikugawa *et al.* 1994).

Dihydropyrimidinase is a tetramer and a Zn-metalloenzyme (Brooks *et al.* 1979, 1983). The Zn cation can be removed by incubation with the chelators *O*-phenanthroline, 8-hydroxyquinoline-5-sulphonic acid, or dipicolinic acid, yielding an inactive apoenzyme (Brooks *et al.* 1979, 1983; Lee *et al.* 1986). The enzyme activity can be restored by

incubating the apoenzyme with Zn (Brooks *et al.* 1979). Zn also effectively inhibits the enzyme activity with a K_i of 23.1 μM (Kikugawa *et al.* 1994). These results suggest that Zn acts as an inhibitor as well as an activator of the enzyme activity.

In the present study, we calculated the period of the cycle of feed intake in rats on a Zn-deficient diet by cosinor analysis. We also found that a Zn-deficient diet results in decrease of rat liver dihydropyrimidinase with diminution of the enzyme protein.

MATERIALS AND METHODS

Animals and diets

Male albino rats (Wistar strain, weighing 100–110 g) were housed in individual screen-bottomed cages in a room maintained at $23 \pm 1^\circ$ with 50% humidity, under controlled lighting conditions (lights on 07.00 to 19.00 hours and lights off 19.00 to 07.00 hours, local time). The animals were fed on a commercial stock diet (Oriental Yeast Ltd., Tokyo, Japan) and water *ad lib.* for 1 week before the experiment to acclimate them to the new environment. Acclimated rats showing progressive weight gain were selected and separated into groups. Body weight and feed intake were determined daily at 09.00 to 10.00 hours. Ten rats were assigned to each experimental group. The rats were given the experimental diet for 4 weeks and killed between 09.00 and 11.00 hours on day 28 under anaesthesia with diethyl ether.

Blood samples were collected into plain glass tubes and stored at 4° overnight until centrifugation at 1500 g for 20 min. Serum samples were stored at -20° until analysis. The liver and kidneys were also removed, weighed and stored at -20° .

The compositions of the Zn-deficient and control diets are shown in Table 1. The contents of Zn in the Zn-deficient and control diets were 0.95 and 45 mg/kg diet respectively. All rats were given deionized water *ad lib.*

Zinc content

A 1 g portion of each test diet, liver and kidney was heated for 48–72 h in a muffle oven at 450° . After the samples cooled, 2 ml 1 M-HCl was added, and the digestates were heated and diluted with doubly-distilled deionized water. Serum was diluted 1:3 with the deionized water.

The concentration of stock Zn and sample solutions was measured by atomic absorption spectroscopy with a Hitachi 208 Atomic Absorption Spectrophotometer (Hitachi Ltd, Tokyo, Japan) at 213.8 nm.

Chemicals

All chemicals used were analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise stated. NADP⁺, 5,6-dihydrouracil and *N*-carbamoyl- β -alanine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Glutamate dehydrogenase (EC 1.4.1.3) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany), and sodium phenyl phosphate from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 5-Bromo-5,6-dihydrouracil was synthesized according to the method described by Zee-Cheng *et al.* (1961).

Enzyme assays

Dihydropyrimidinase. The liver was homogenized in 10 volumes of 10 mM-potassium phosphate, pH 7.0, containing 10 mM-2-mercaptoethanol. After centrifugation, the supernatant fraction was used for analysis of dihydropyrimidinase and β -ureidopropionase (EC 3.5.1.6) activities.

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

	Control	Zinc-deficient
Egg albumin	200	200
Dextrin	637	637
Maize oil	100	100
Vitamin mixture*	11.7	11.7
Salt mixture (+ zinc)†	31.3	—
Salt mixture (– zinc)‡	—	31.3
Cellulose powder	20	20

* Vitamins were (mg/kg diet): retinyl acetate 2.01, cholecalciferol 0.03, α -tocopheryl acetate 58.5, menadione 60.8, thiamine hydrochloride 14.0, riboflavin 46.8, pyridoxine hydrochloride 9.4, cyanocobalamin 0.006, ascorbic acid 351, D-biotin 0.23, pteroylglutamic acid 2.34, calcium pantothenate 58.5, *p*-aminobenzoic acid 58.5, niacin 70.2, choline chloride 2340.

† Minerals (+ zinc) were (g/kg diet): $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ 4.557, KH_2PO_4 8.050, NaH_2PO_4 2.927, NaCl 1.459, Ca-lactate 10.983, Fe-citrate 0.995, MgSO_4 2.244, ZnCO_3 0.034, $\text{MnSO}_4 \cdot 4 \sim 6\text{H}_2\text{O}$ 0.038, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.009, KI 0.003.

‡ ZnCO_3 was omitted from the above mixture.

Dihydropyrimidinase activity was determined by measuring the rate of decrease in absorbance associated with 5-bromo-5,6-dihydrouracil at 225 nm in a cuvette with a 1.0 cm light path at 37° (Brooks *et al.* 1983). The standard reaction mixture contained 50 mM-Tris-HCl buffer, pH 8.2, and 0.17 mM-5-bromo-5,6-dihydrouracil in a total volume of 3.0 ml.

β -Ureidopropionase. β -Ureidopropionase activity was measured with respect to the rate of formation of NH_3 (Tamaki *et al.* 1987). The standard reaction mixture contained 0.1 M-sodium phosphate, pH 7.0, including bovine serum albumin (1 g/l), 10 mM-MgCl₂, 1 mM-EDTA, 5 mM-2-mercaptoethanol and 2 mM-*N*-carbamoyl- β -alanine. Incubation was carried out in a shaking water bath for 30 min at 37°.

Dihydropyrimidine dehydrogenase. The liver was homogenized in 10 volumes of 10 mM-potassium phosphate, pH 7.4, containing 5 mM-2-mercaptoethanol and 2.5 mM-MgCl₂. After centrifugation, the supernatant fraction was heated to 50° for 1 min and then cooled to 4°. The precipitate was discarded after centrifugation and the supernatant fraction was adjusted to pH 4.85 with acetic acid (50 ml/l). After centrifugation, the supernatant fraction was neutralized with 0.5 M-KOH and treated with $(\text{NH}_4)_2\text{SO}_4$. The precipitate obtained at 30–50% saturation was dissolved in a minimum volume of the above buffer and used for enzyme analysis.

Dihydropyrimidine dehydrogenase (*EC* 1.3.1.1) activity was followed by measuring the rate of disappearance of NADPH at 37° (Fritzson, 1960). The standard assay mixture contained 50 mM-potassium phosphate, pH 7.4, including 0.15 mM-uracil and 0.15 mM-NADPH, in a total volume of 3.0 ml.

Alkaline phosphatase. Alkaline phosphatase (*EC* 3.1.3.1) activity was measured with respect to the rate of formation of phenol from sodium phenyl phosphate (Bessey *et al.* 1946). The absorbance at 405 nm was measured.

Preparation of antiserum

Purified dihydropyrimidinase (0.7 mg) from rat liver was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously twice into a rabbit at 2-week intervals. Two weeks after the second injection, 0.2 mg of the enzyme was injected intravenously. The rabbit was bled 2 weeks after intravenous injection and the serum

collected. Control serum was prepared from rabbits before the first injection of the purified enzyme.

Immunodiffusion and immunoprecipitation

Double immunodiffusion was performed in agar (10 g/l) in 20 mM-potassium phosphate (pH 7.2).

In the immunological quantitation of dihydropyrimidinase, a total volume of 0.3 ml (various amounts of sample solution and 5 μ l antiserum, adjusted to a fixed volume with 20 mM-potassium phosphate, pH 7.2, containing 0.225 M-KCl) was incubated for 30 min at 37°, then allowed to stand for 16 h at 4°. After addition of 0.3 ml protein A-Sepharose CL-4B suspended in phosphate-buffered saline (4.0 g NaCl, 0.1 g KCl, 1.45 g Na₂HPO₄ · 12H₂O and 0.1 g KH₂PO₄, in a total volume of 500 ml by addition of H₂O), the reaction mixture was allowed to stand for 60 min at 25°, before a brief centrifugation. The supernatant fraction was assayed for dihydropyrimidinase activity.

Derivation of feed intake and body-weight change

Feed intake data and body-weight change data were analysed by a modification of the 'Cosinor' method (Halberg *et al.* 1972). Feed intake (F) or body-weight change (ΔB) at day t was derived from the following equation:

$$F(\text{or } \Delta B) = M + A \cos(2\pi(t - \psi)/\tau),$$

where M , A , τ and ψ represent mesor (the rhythm-adjusted mean), amplitude (maximum or minimum value from the adjusted mean), period (the length of one complete cycle), and phase shift (time of maximum on the first cycle) respectively.

The experimental data obtained were fitted to the above equation by a nonlinear least-squares method. The four parameters M , A , τ and ψ were calculated in the analysis (Marquardt, 1963), and are given with standard errors, using a subroutine containing an SALS, a computer program which was developed by the computer centre at the University of Tokyo (Nakagawa & Oyanagi, 1980).

Protein measurement

Protein concentration was measured with Folin phenol reagent (Lowry *et al.* 1951), using bovine serum albumin as a standard.

Statistical analysis

One-way analysis of variance was used to compare the groups. When a significant difference ($P < 0.05$) was found between groups, the statistical significance of difference between values was assessed by Student's t test.

RESULTS

Body weight and feed intake of rats fed on zinc-deficient diets

The Zn-deficient rats showed the typical symptoms of Zn deficiency such as alopecia, depigmentation of hair, dermatitis of the paws, and anorexia with growth retardation, which have been described by Wallwork *et al.* (1981). Fig. 1 shows the average weight gain in each group. The body weight of the control rats increased linearly under experimental conditions, while that of the Zn-deficient animals was retarded in a cyclic pattern. The final weight gains of the rats fed on control and Zn-deficient diets for 4 weeks were 144.6 (SE 5.3) and 38.2 (SE 2.3) g respectively.

The feed intake in the Zn-deficient diet group was 64.1% of the intake in the control

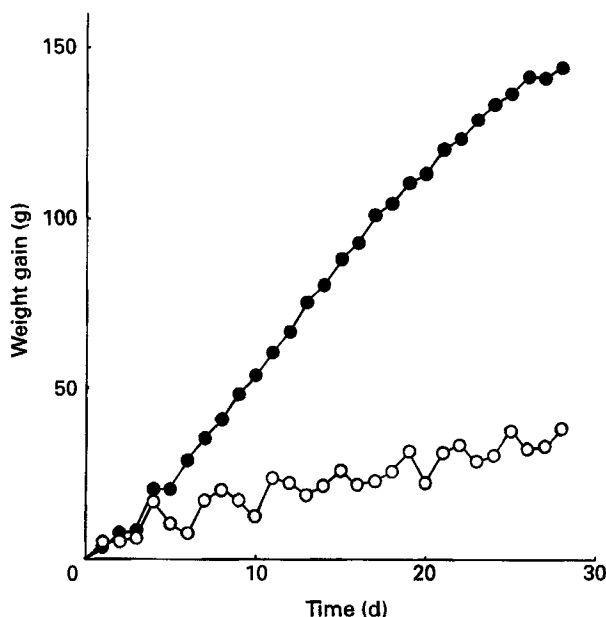


Fig. 1. Average weight gain over time in rats fed on a control diet (●) or a zinc-deficient diet (○).

group. Fig. 2a illustrates the cyclic feeding pattern of five Zn-deficient rats, which has been described by others (Mills *et al.* 1969; Chesters & Quarterman, 1970; Wallwork *et al.* 1981; Kramer *et al.* 1984). The values of the rhythmometric parameters obtained by Cosinor analysis are shown in Table 2. There was a cyclic variation in feed intake in each of the five rats as well as in the group (Table 2 and Fig. 2). The cyclical period of the feed intake of the Zn-deficient rats ranged from 3.3 to 3.6 d, and the phase shift was from 0.7 to 1.3 d.

The mean values of M , A , τ and ψ for the five Zn-deficient rats were calculated to be 10.1 (SE 0.4) g/d, 3.5 (SE 0.5) g/d, 3.5 (SE 0.1) d and 1.0 (SE 0.1) d respectively.

Cosinor analysis demonstrated that the pattern of body change over 28 d could also be fitted to a cosine wave with very high correlation (Fig. 2b and Table 3). The period of the cycle ranged from 3.3 to 3.7 d and was closely related to that of the intake of the Zn-deficient diet. However, the phase shift (time of maximum on the first cycle) of the cycle for body-weight change was 0.5 d shorter than that of the cycle for the feed intake. The calculated mean values of M , A , τ and ψ for individual means in the Zn-deficient rats were 1.4 (SE 0.1) g/d, 7.9 (SE 1.3) g/d, 3.5 (SE 0.1) d and 0.5 (SE 0.1) d respectively.

The acrophase of the cycle could be represented by the formula $2\pi(\tau - \psi)/\tau$. The calculated mean values of acrophase for the cycle of feed intake and weight change were $(1.44 \pm 0.07)\pi$ and $(1.73 \pm 0.07)\pi$ radian respectively.

Serum and liver zinc content in zinc-deficient rats

The mean daily Zn intakes calculated from feed intake in the control and Zn-deficient diet groups were 708 (SE 17) and 9.1 (SE 0.45) μg respectively. The serum Zn content showed a significant decrease during the course of the Zn depletion in the latter group. At 4 weeks the serum Zn contents in the two groups were 2.58 (SE 0.09) and 0.81 (SE 0.07) mg/ml respectively ($P < 0.01$). The alkaline phosphatase activities in the serum of the rats fed on the control and Zn-deficient diets were 9.62 (SE 0.54) and 4.92 (SE 1.12) nmol/min per mg protein respectively ($P < 0.01$).

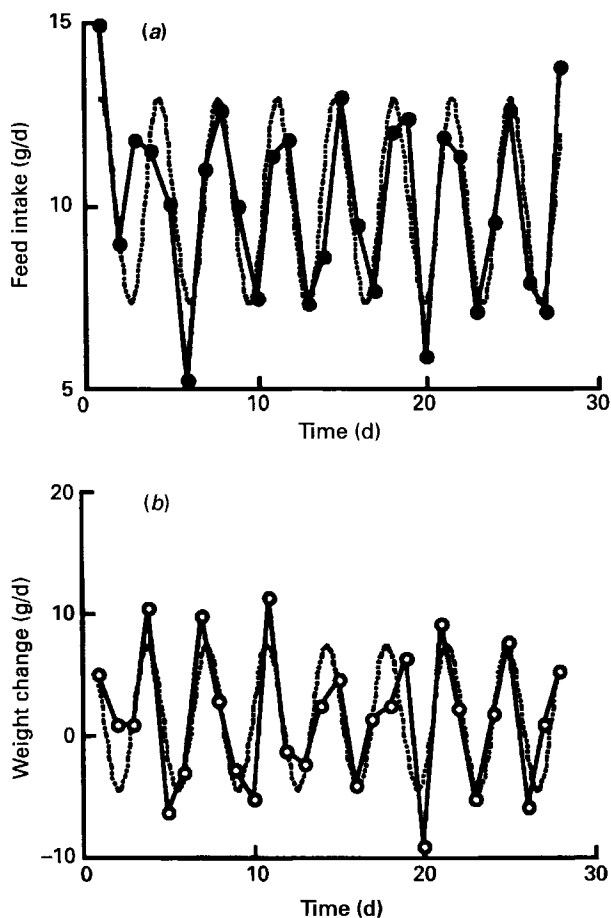


Fig. 2. Pattern of cyclic variation of feed intake (a) and body-weight change (b) of rats fed on a zinc-deficient diet. Each point represents the average feed intake (a, ●) or body-weight change (b, ○) for the previous 24 h period. All the data from five rats were analysed by the Cosinor method. (---), The curve with best fit obtained with computer-generated cosine curves. $F = 10.1 + 2.8 \cos(2\pi(t-1.0)/3.4)$. $\Delta B = 1.4 + 5.9 \cos(2\pi(t-0.5)/3.5)$.

Table 2. *Rhythmometric summary of Cosinor analysis of zinc-deficient-feed intake in the previous 24 h period of rats for 28 d*

(Values with their standard errors)

Rat...	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5	
	Value	SE	Value	SE	Value	SE	Value	SE	Value	SE
Mesor (M^*) (g/d)	10.4	0.2	11.2	1.6	9.4	0.1	10.2	0.2	9.2	0.7
Amplitude (A) (g/d)	4.5	0.3	3.1	2.2	3.8	0.3	1.6	0.3	4.5	0.9
Period (τ) (d)	3.4	0.1	3.6	0.5	3.3	0.1	3.5	0.1	3.5	0.1
Phase shift (ψ) (d)	1.3	0.1	0.8	0.7	1.2	0.1	0.7	0.2	0.9	0.1
R^2	0.611		0.401		0.464		0.275		0.617	
P	< 0.0001		< 0.0005		< 0.0001		< 0.005		< 0.0001	

* Rhythm-adjusted mean.

Feed intake in the previous 24 h period at a t day (F): $F = M + A \cos(2\pi(t-\psi)/\tau)$.

Time of maximum feed intake in the previous 24 h period (d) = $n\tau + \psi$ ($n = 0, 1, 2, 3, \dots$).

Time of minimum feed intake in the previous 24 h period (d) = $(n + 1/2)\tau + \psi$ ($n = 0, 1, 2, 3, \dots$).

Table 3. *Rhythmometric summary of Cosinor analysis of weight change in the previous 24 h period of rats fed on a zinc-deficient diet for 28 d*

(Values with their standard errors)

Rat...	Rat 1		Rat 2		Rat 3		Rat 4		Rat 5	
	Value	SE	Value	SE	Value	SE	Value	SE	Value	SE
Mesor (M^*) (g/d)	1.4	0.2	1.7	0.2	0.9	0.2	1.4	0.2	1.4	0.2
Amplitude (A) (g/d)	7.8	0.3	9.1	0.3	10.1	0.3	3.1	0.3	9.5	0.3
Period (τ) (d)	3.4	0.1	3.7	0.1	3.3	0.1	3.5	0.1	3.5	0.1
Phase shift (ψ) (d)	0.8	0.05	0.3	0.03	0.7	0.03	0.2	0.11	0.4	0.04
R^2	0.479		0.401		0.430		0.163		0.653	
P	< 0.0001		< 0.0005		< 0.0002		< 0.05		< 0.0001	

* Rhythm-adjusted mean.

Weight change in the previous 24 h period at a t day (ΔB): $\Delta B = M + A \cos(2\pi(t - \psi)/\tau)$.

Time of maximum weight change in the previous 24 h period (d) = $n\tau + \psi$ ($n = 0, 1, 2, 3 \dots$).

Time of minimum weight change in the previous 24 h period (d) = $(n + 1/2)\tau + \psi$ ($n = 0, 1, 2, 3 \dots$).

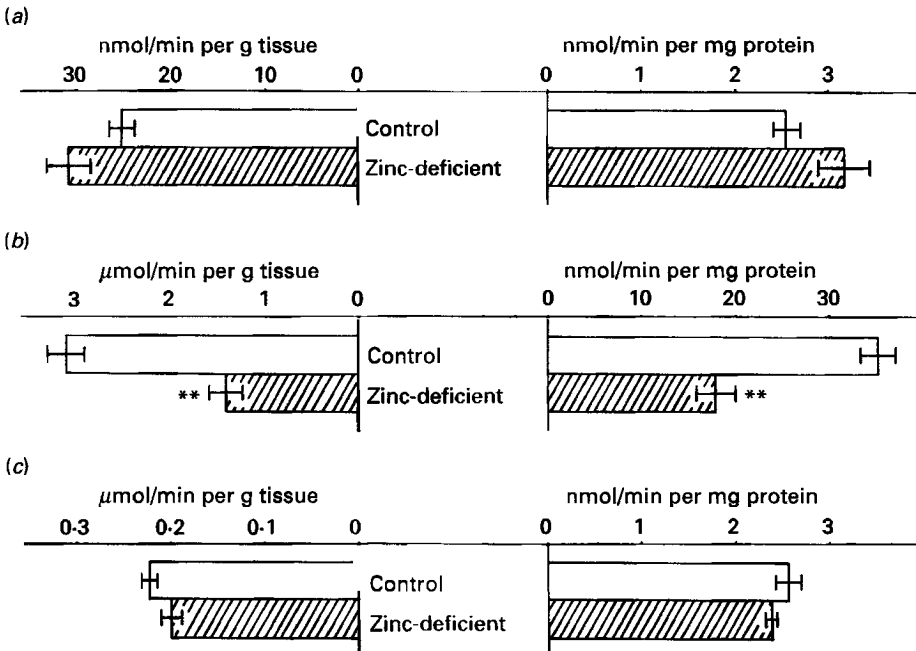


Fig. 3. Effect of a zinc-deficient diet on the activities of rat liver pyrimidine-metabolizing enzymes: (a) dihydropyrimidine dehydrogenase ($EC\ 1.3.1.1$), (b) dihydropyrimidinase ($EC\ 3.5.2.2$) and (c) β -ureido-propionase ($EC\ 3.5.1.6$). The protein concentration in the precipitate obtained at 30–50% ammonium sulphate saturation (a) and in the supernatant fraction after centrifugation of the homogenate (b and c) was estimated as described on p. 714. Values are means with their standard errors indicated by vertical bars. (□), Control group; (▨), zinc-deficient group. ** Mean value was significantly different from that of the control group, $P < 0.01$.

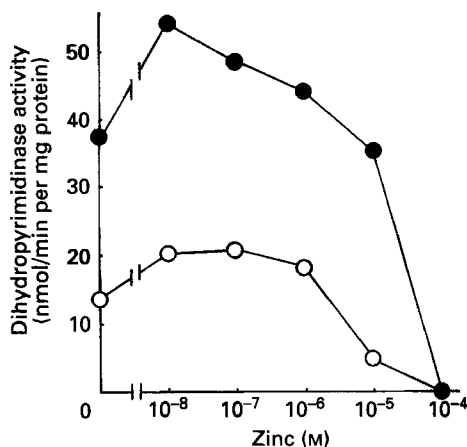


Fig. 4. Effect of addition of exogenous zinc on rat liver dihydropyrimidinase (*EC* 3.5.2.2) activity *in vitro*. Rat liver (1 g tissue, 0.2 g from each of five different rats) was homogenized in 10 ml 10 mM-potassium phosphate, pH 7.0, containing 10 mM-2-mercaptoethanol. The homogenate was centrifuged for 20 min at 27000 g. The supernatant fraction was used as sample solution. The enzyme activity was assayed by exogenous addition of $Zn(NO_3)_2$ at the indicated zinc concentration. (●), Control group; (○), zinc-deficient diet group.

The liver Zn level in contrast to the serum level, was not markedly affected by the Zn-deficient diet. The mean Zn contents of the liver at 4 weeks in the control and Zn-deficient groups were 19.52 (SE 1.34) and 16.42 (SE 0.85) $\mu\text{g/g}$ tissue respectively.

Effect of zinc-deficient diet on pyrimidine-catabolizing enzymes

Fig. 3 shows the activities of dihydropyrimidine dehydrogenase, dihydropyrimidinase and β -ureidopropionase in the control and Zn-deficient groups. Of these three enzymes in the rat liver, dihydropyrimidinase activity was significantly decreased by the Zn-deficient diet. However, the activities of these enzymes in the kidney were not affected by the Zn-deficient diet (results not shown).

When the value of $\cos(2\pi(t-\psi)/\tau)$ at day 28 in the formula of the feed intake in rats fed on the Zn-deficient diet was simulated, we could not find any relationship between the cosine value and the liver dihydropyrimidinase activity. These results suggest that dihydropyrimidinase activity in liver of Zn-deficient rats has no cyclic variation.

Effect of exogenous addition of zinc on dihydropyrimidinase activity

Fig. 4 shows the effect of exogenous Zn addition on rat liver dihydropyrimidinase activity. After addition of 10 nM-Zn to liver crude extract from control and Zn-deficient rats, the dihydropyrimidinase activity showed 1.44- and 1.50-fold activation respectively, compared with that without Zn addition. However, a high concentration of Zn (0.1 mM) completely inactivated the enzyme in both the control and Zn-deficient groups.

Quantitation of antigen by immunotitration

The antiserum to rat liver dihydropyrimidinase produced a single connecting band of precipitin between purified dihydropyrimidinase and crude extract from rat liver (Ouchterlony double diffusion). The antiserum inhibited enzyme activity of the crude extracts from control and Zn-deficient rat liver. The quantitative immunotitration results are shown in Fig. 5. It can be seen that the equivalence points for the titrations were 0.26 mg and 0.52 mg of crude extract from control and Zn-deficient rat liver respectively, per 5 μl

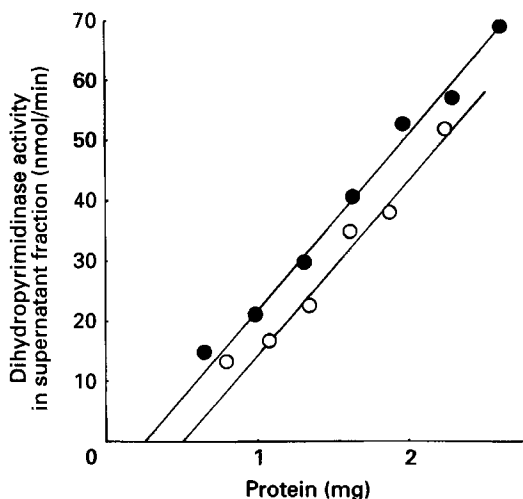


Fig. 5. Immunological quantitation of dihydropyrimidinase (*EC* 3. 5. 2. 2) activity in zinc-deficient and control rat liver supernatant fractions. Rat liver (1 g tissue, 0.2 g from each of five different rats) was homogenized in 10 volumes of 10 mM-potassium phosphate, pH 7.0, containing 10 mM-2-mercaptoethanol. The homogenate was centrifuged for 20 min at 27000 *g*. The supernatant fraction was used as sample solution. The specific activities of dihydropyrimidinase in samples from control and zinc-deficient rat liver were 32.4 and 12.6 nmol/min per mg protein respectively. After titration with antiserum, the supernatant fluids were assayed for dihydropyrimidinase activity. (●), Control group; (○), zinc-deficient group.

antibody. It can be calculated that 1 μ l antiserum titrated 1.7 nmol/min dihydropyrimidinase in the liver from control as well as Zn-deficient rats.

DISCUSSION

In the present study the young male rats fed on a Zn-deficient diet showed the typical signs of Zn deficiency, including anorexia with marked decrease of growth compared with control rats. This reduction in feed intake was previously reported to be accompanied by a pattern of cyclic feeding in rats with Zn-deficient diet (Mills *et al.* 1969; Chesters & Quarterman, 1970; Chesters & Will, 1973; Wallwork *et al.* 1981; Gingliano & Millward, 1984; Kramer *et al.* 1984; Quinn *et al.* 1990). Here, we found by Cosinor analysis that the period of the cycle was 3.5 (SE 0.1) d. These results suggest that rats fed on Zn-deficient diet show little variation in feeding pattern, which consistently shows a cycle of 3.5 d.

When the feed intake of rats is examined at 2 h intervals throughout the day, Zn-deficient rats are found to eat on fewer occasions than control rats (Chesters & Will, 1973). However, during those 2 h periods when the Zn-deficient rats did eat, the quantities eaten in 2 h showed the same weight distribution as those for the Zn-adequate rats (Chesters & Will, 1973). When we examined the cycle of feed intake and body-weight change of Zn-deficient rats for each 24 h period throughout the 4-week study, we found both had a 3.5 (SE 0.1) d period of the cycle by computer Cosinor analysis.

Zn deficiency impairs growth by a combination of reduced feed intake, reduced anabolic response to feed, and increased catabolic response to the reduced intake in which corticosterone may play a role (Gingliano & Millward, 1987). Recently, it was proposed that the growth retardation seen in Zn-deficient states may be mediated through reduced serum-insulin-like growth factor-1 production (Dorup *et al.* 1991). However, we could not account for the 3.5 d cycle of feed intake and body change through these effectors.

The period of the cycle of feed intake was in good agreement with that of the body-

weight change. However, we found marked differences between the amplitudes of the cycle. The feed intake values are for essentially dry weights of diet whereas the body-weight changes represent differences in wet weight. If the tissues being lost and gained in a cyclical manner contain, say, 80% or more water, the differences could largely be explained on this basis. In fact, daily intakes of water and feed are closely correlated for the Zn-deficient rats (Wallwork *et al.* 1981). Therefore, the amplitude in body weight may be about twice that in feed intake.

Dihydropyrimidinase is a Zn-metalloenzyme, containing four tightly bound Zn ions per molecule of active enzyme (Brooks *et al.* 1971, 1983). The Zn-deficient diet significantly inactivated dihydropyrimidinase in liver with a reduction of the enzyme protein. However, the activity of β -ureidopropionase, which contains two putative Zn-binding site motifs (Kvalnes-Krick & Traut, 1993), was maintained at the control level in the rats fed on the Zn-deficient diet, while dihydropyrimidine dehydrogenase activity showed a slight increase. These results suggest that rat liver dihydropyrimidinase activity is more strongly affected by Zn deficiency than is β -ureidopropionase. Since protein synthesis and RNA concentration are reduced in the Zn-deficient rat (Gingliano & Millward, 1987), dihydropyrimidinase may be regulated only under physiological Zn conditions.

The rate-limiting enzyme in pyrimidine catabolism in the rat liver is dihydropyrimidine dehydrogenase (Canellakis, 1956; Fritzson, 1957). However, in normal human extrahepatic tissues, dihydropyrimidinase, rather than dihydropyrimidine dehydrogenase, is the rate-limiting enzyme in the pyrimidine base catabolic pathway (Naguib *et al.* 1985). The dihydropyrimidinase level in solid tumours is increased compared with that in normal tissues (Naguib *et al.* 1985). During the catabolism of 5-fluorouracil in isolated rat hepatocytes, the predominant metabolite is 5-fluoro-5,6-dihydrouracil (Sommadosi *et al.* 1982), and the rate-limiting step which has been identified by a mathematical model of the kinetics is the degradation of 5-fluorodihydrouracil (Mentre *et al.* 1984). These findings suggest that dihydropyrimidinase has an important function in pyrimidine metabolism.

The active form of dihydropyrimidinase from rat liver contains four Zn ions per molecule, presumably at the ratio of one per subunit (Brooks *et al.* 1979, 1983; Kikugawa *et al.* 1994). The Zn can be removed by incubation with chelators, and the enzyme activity can be restored by incubation of the apoenzyme with $5 \mu\text{M}$ -Zn (Brooks *et al.* 1983). However, Zn also acts as an irreversible inhibitor with a K_i of $23.1 \mu\text{M}$ (Kikugawa *et al.* 1994). Therefore, Zn acts as an inhibitor as well as an activator of the enzyme *in vitro*. The total level of Zn in the liver was calculated to be 0.3 mM. However most of the Zn present *in vivo* is tightly bound and its concentration is little altered by Zn deficiency. Therefore, it is impossible that Zn acts as an inhibitor of dihydropyrimidinase under physiological conditions.

The immunological analysis revealed that both the activity and the protein concentration of dihydropyrimidinase in the liver of rats fed on the Zn-deficient diet were lower than those of control rats. The addition of Zn to the extract from Zn-deficient rat liver did not increase the activation to the control level. These results indicate that the level of apo-form of the enzyme is not increased in the Zn-deficient rat and that the total enzyme protein may be regulated by Zn. Nevertheless, the function of Zn in dihydropyrimidinase concentration requires further detailed study.

REFERENCES

- Bessey, O. A., Lowry, O. H. & Brock, M. J. (1946). A method for rapid determination of alkaline phosphatase with 5 cubic millimeters of serum. *Journal of Biological Chemistry* **164**, 321–329.
- Brooks, K. P., Jones, E. A., Kim, B.-D. & Sander, E. G. (1983). Bovine liver dihydropyrimidine amidohydrolase; purification, properties and characterization as a zinc metalloenzyme. *Archives of Biochemistry and Biophysics* **226**, 469–483.

- Brooks, K. P., Kim, B.-D. & Sander, E. G. (1979). Dihydropyrimidine amidohydrolase is a zinc metalloenzyme. *Biochimica et Biophysica Acta* **570**, 213–214.
- Canellakis, E. S. (1956). Pyrimidine metabolism. I. Enzymatic pathways of uracil and thymine degradation. *Journal of Biological Chemistry* **221**, 315–322.
- Chesters, J. K. & Quarterman, J. (1970). Effects of zinc deficiency on food intake and feeding patterns of rats. *British Journal of Nutrition* **24**, 1061–1069.
- Chesters, J. K. & Will, M. (1973). Some factors controlling food intake by zinc-deficient rats. *British Journal of Nutrition* **30**, 555–566.
- Dorup, I., Flyvbjerg, A., Everts, M. A. & Clausen, T. (1991). Role of insulin-like growth factor-1 and growth hormone in growth inhibition induced by magnesium and zinc deficiencies. *British Journal of Nutrition* **66**, 505–521.
- Droke, E. A., Spears, J. W., Armstrong, J. D., Kegley, E. B. & Simpson, R. B. (1993). Dietary zinc affects serum concentrations of insulin and insulin-like growth factor I in growing lambs. *Journal of Nutrition* **123**, 13–19.
- Fritzson, P. (1957). The catabolism of C¹⁴-labeled uracil, dihydrouracil, and β -ureidopropionic acid in rat liver slices. *Journal of Biological Chemistry* **226**, 223–228.
- Fritzson, P. (1960). Properties and assay of dihydrouracil dehydrogenase of rat liver. *Journal of Biological Chemistry* **235**, 719–725.
- Gingliano, R. & Millward, D. J. (1984). Growth and zinc homeostasis in the severely Zn-deficient rat. *British Journal of Nutrition* **52**, 545–560.
- Gingliano, R. & Millward, D. J. (1987). The effects of severe zinc deficiency on protein turnover in muscle and thymus. *British Journal of Nutrition* **57**, 139–155.
- Golden, B. E. (1988). Zinc in cell division and tissue growth: physiological aspects. In *Zinc in Human Biology*, pp. 119–128 [C. F. Mills, editor]. Berlin: Springer Verlag.
- Halberg, F., Johnson, E. A., Nelson, W., Runge, W. & Sothorn, R. (1972). Autorhythmometry-procedures for physiologic self-measurements and their analysis. *Physiology Teacher* **1**, 1–11.
- Kautz, J. & Schnackerz, K. D. (1989). Purification and properties of 5,6-dihydropyrimidine amidohydrolase from calf liver. *European Journal of Biochemistry* **181**, 431–435.
- Kikugawa, M., Kaneko, M., Fujimoto-Sakata, S., Maeda, M., Kawasaki, K., Takagi, T. & Tamaki, N. (1994). Purification, characterization and inhibition of dihydropyrimidinase from rat liver. *European Journal of Biochemistry* **219**, 393–399.
- Kramer, T. R., Briske-Anderson, M., Johnson, S. B. & Holman, R. T. (1984). Influence of reduced food intake on polyunsaturated fatty acid metabolism in zinc-deficient rats. *Journal of Nutrition* **114**, 1224–1230.
- Kvalnes-Krick, K. L. & Traut, T. W. (1993). Cloning, sequencing, and expression of a cDNA encoding β -alanine synthase from rat liver. *Journal of Biological Chemistry* **268**, 5686–5693.
- Lee, M. H., Cowling, R. A., Sander, E. G. & Pettigrew, D. W. (1986). Bovine liver dihydropyrimidine amidohydrolase: pH dependencies of inactivation by chelators and steady-state kinetic properties. *Archives of Biochemistry and Biophysics* **248**, 368–378.
- Lee, M. H., Pettigrew, D. W., Sander, E. G. & Nowak, T. (1987). Bovine liver dihydropyrimidine amidohydrolase: pH dependencies of the steady-state kinetic and proton relaxation rate properties of the Mn(II)-containing enzyme. *Archives of Biochemistry and Biophysics* **259**, 597–604.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- Maguire, J. H. & Dudley, K. H. (1978). Partial purification and characterization of dihydropyrimidinase from calf and rat liver. *Drug Metabolism and Disposition* **6**, 601–605.
- Marquardt, D. W. (1963). An algorithm for least-squared estimation of nonlinear parameters. *Journal of the Society for Industrial and Applied Mathematics* **11**, 431–441.
- Mentre, F., Steimer, J.-L., Sommadossi, J.-P., Diasio, R. B. & Cano, J.-P. (1984). A mathematical model of the kinetics of 5-fluorouracil and its catabolites in freshly isolated rat hepatocytes. *Biochemical Pharmacology* **33**, 2727–2732.
- Mills, C. F., Quarterman, J., Chesters, J. K., Williams, R. B. & Dalgarno, A. G. (1969). Metabolic role of zinc. *American Journal of Clinical Nutrition* **22**, 1240–1249.
- Naguib, F. N. M., el Kouni, M. H. & Cha, S. (1985). Enzymes of uracil catabolism in normal and neoplastic human tissues. *Cancer Research* **45**, 5405–5412.
- Nakagawa, T. & Oyanagi, Y. (1980). Program system SALS for nonlinear least squares fitting in experimental sciences. In *Recent Development in Statistical Inference and Data Analysis*, pp. 221–225. [K. Matsushita, editor]. Amsterdam: North-Holland Publishing Co.
- Quinn, P. B., Cremin, F. M., O'Sullivan, V. R., Hewedi, F. M. & Bond, R. J. (1990). The influence of dietary folate supplementation on the incidence of teratogenesis in zinc-deficient rats. *British Journal of Nutrition* **64**, 233–243.
- Sommadossi, J.-P., Gewirtz, D. A., Diasio, R. B., Aubert, C., Cano, J.-P. & Goldman, I. D. (1982). Rapid catabolism of 5-fluorouracil in freshly isolated rat hepatocytes as analyzed by high performance liquid chromatography. *Journal of Biological Chemistry* **257**, 8171–8176.
- Tamaki, N., Mizutani, N., Kikugawa, M., Fujimoto, S. & Mizota, C. (1987). Purification and properties of β -ureidopropionase from the rat liver. *European Journal of Biochemistry* **169**, 21–26.

- Wallach, D. P. & Grisolia, S. (1957). The purification and properties of hydropyrimidine hydrase. *Journal of Biological Chemistry* **226**, 277–288.
- Wallwork, J. C., Fosmire, G. J. & Sandstead, H. H. (1981). Effect of zinc deficiency on appetite and plasma amino acid concentrations in the rat. *British Journal of Nutrition* **45**, 127–136.
- Williams, R. B. & Mills, C. F. (1970). The experimental production of zinc deficiency in the rat. *British Journal of Nutrition* **24**, 989–1003.
- Zee-Cheng, K.-Y., Robins, R. K. & Cheng, C. C. (1961). Pyrimidines. III. 5,6-Dihydropyrimidines. *Journal of Organic Chemistry* **26**, 1877–1884.