Idiorrhythmic dose-rate variability in dietary zinc intake generates a different response pattern of zinc metabolism than conventional dose–response feeding*†

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We compared the effects of idiorrhythmic dose-rate feeding and conventional dose–response on the induction of intestinal metallothionein (iMT), expression of aortal heat-shock protein mRNA (HSP70mRNA) induced by restraint stress, and accumulation of Zn in the femur and incisor of young growing male rats. An idiorrhythmic approach requires that the average dietary Zn concentration (modulo, M) over the whole experiment (epoch, E) is kept constant across different groups. This is done by adjusting the Zn concentration of the supplemented diet supplied to compensate for the reduction in the number of days on which Zn-supplemented diet is fed, the latter being spread evenly over the experiment. Idiorrhythms involve offering the diet with *n* times the overall Zn concentration (M) only every *n*th day with Zn-deficient diet offered on other days. Idiorrythmic Zn dose-rate feeding changed Zn accumulation in the femur and incisor in a complex bi-modal fashion, indicating that metabolic efficiency of dietary Zn is not constant but depends on Zn dose-rate. In contrast to feeding Zn in the conventional dose–response scheme, iMT and HSP70mRNA were not affected by idiorrhythmic dose-rate feeding. Idiorrhythmic cycling in dietary Zn load posed no risk of a biochemical overload nor caused the animals to be stressed. Idiorrhythmic dose-rate feeding brings the dimension of time to the conventional dose–response model.

Zinc: Dose-rate idiorrhythm: Intestinal metallothionein: Calcified tissue

Our knowledge of essential nutrient requirements and metabolism is based primarily on experiments where animals are fed on a constant diet, although a constant diet is rarely, if ever, consumed in real life. The broader health and metabolic consequences of natural temporal variability in food intake (Klevay, 1984) and especially that of dose-rate (Momčilović, 1988), are largely unknown. This is surprising, as nutrition may be viewed as a life-long series of time-discrete events.

The habitual consumption of trace elements like Zn varies widely among individuals, and daily ingestion of Zn supplements only adds to this natural variability (Smith, 1994). Mertz (1993) reported that the variability in dietary Zn consumption results in different

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metabolic pool sizes and, therefore, as a result of homeostasis, the quantity of Zn necessary to maintain the existing pool of body Zn will vary. It was recently shown by Viteri *et al.* (1995) that variability in nutrient intake, like partitioning between different days, may have important metabolic consequences. They found that true absorption and retention of supplemental Fe was more efficient when administered to normal and Fe-deficient rats every 3 d rather than daily. Similarly, Gleerup *et al.* (1995) compared the effect of two different distributions of daily Ca intake on Fe absorption from the whole diet and concluded that a reasonable separation of Ca and Fe intakes between meals on the same day would improve Fe nutrition.

The logic of cycles and other temporal effects is not the same as the logic of linear and monotonic trends (Kelly & McGrath, 1988). Recently, an idiorrhythmic feeding regimen was proposed to study the impact of dose-rate in trace element nutrition (Momčilović, 1995). In contrast with the conventional dose–response feeding, the idiorrhythmic dose-rate feeding mimics natural conditions in a controllable way, i.e., if and when the nutrient is available. This model is based on offering a constant total dose (dose–time equivalent modulo) to animals by inversely correlating the amount and frequency of individual doses (idiorrhythm) over a selected period of time (epoch, E). Viewing the dose as a dose–time equivalent over the time span of an entire experimental E allows one to divide the dose into a series of equal products of different doses with different frequencies. Then they are administered in a regularly recurring pattern to allow the same total dose as if the animals were conventionally fed with Zn every day, albeit at a different rate. Therefore, idiorrhythmic feeding is a rigorously defined model for dose-rate studies and is in clear distinction to other, predominantly descriptive, concepts of meal and nutrient partitioning.

Recently, it was found that the Zn dose-rate idiorrhythm induces changes in metabolic efficiency, which in turn generates a complex bi-modal growth response pattern where an intake of dietary Zn exceeding requirements has a limited capacity to compensate for a previously deficient Zn intake. This capacity is dependent on both the dose–time equivalent modulo and the dose-rate idiorrhythm (Momčilović, 1995).

The aim of the present experiment was to observe the effects of the idiorrhythmic Zn dose-rate feeding on Zn accumulation in the femur and incisor and then compare the results with those of the conventional dose–response model. The femur has already been shown to be the tissue of choice for the assessment of dietary Zn bioavailability. The incisor should be a useful addition in this respect because of its different growth characteristics (Momčilović *et al.* 1975*a,b*). We also wanted to know if the temporal idiorrhythmic increase of Zn load on dosing days is large enough to invoke a biochemical defence response by inducing intestinal metallothionein (iMT), which is thought to function in Zn absorption, homeostasis, and protection from metal toxicity (Dunn *et al.* 1987). Idiorrhythmic dose-rate cycling of dietary Zn load generates repetitive saltatory response of growth-spurts, -cessation, and -catch-up respectively (Momčilović, 1995), and we are concerned that such a strong environmental stress may induce the expression of stress proteins such as aortal heat-shock, HSP70 (Welch, 1992). We think that understanding the biological mode of operation of idiorrhythmic Zn dose-rate feeding may prove helpful in the study of the effects of nutrient partitioning across meals, in the nutri-pharmaceutical properties of foods, and in supplementation programmes (Momčilović, 1995).

MATERIALS AND METHODS

Idiorrhythmic dose-rate experimental feeding design

Idiorrhythm (I) describes a distinctly proportional and regularly recurrent pattern. An idiorrhythmic approach requires that average dietary Zn concentration (modulo, M) over

the whole experiment (epoch, E) is kept constant across different groups. This is done by adjusting the Zn concentration of the supplemented diet supplied to compensate for the reduction in the number of days on which Zn-supplemented diet is fed, the latter being spread evenly over the experiment. Idiorrhythms involve offering the diet with *n* times the overall Zn concentration (M) only every *n*th day with Zn-deficient diet offered on other days. Thus for a protocol involving feeding the Zn-supplemented diet every third day, the diet would contain three times the average Zn concentration and the idiorrhythm would be designated 3M3 (Fig. 1) (Momčilović, 1995). The conventional dose–response model, where the nutrient is fed on a continuous daily basis at a constant dose, may be regarded as a special case of an idiorrhythm where the time base is only 1 d.

In a more formal sense, the relationship between the dose-rate idiorrhythm, the selected dose–time equivalent modulo level (Mx; x = mg Zn/kg per d_1), and the sequential number of the day on which the peak dose is administered, i.e., dosing day (d_{nth}), is expressed as: $I = [d_{nth}(Mx)]/d_{nth}$. All of the idiorrhythms that share the same Zn dosing day (d_{nth}) are considered analogous regardless of their Zn dose–time equivalent modulo level (Mx). To facilitate the comparison of such analogous idiorrhythms, Mx is added as a subscript (I_{Mx}).

Experimental diets

The composition of the basal Zn-deficient diet is shown in Table 1 (Momčilović *et al.* 1976). It was an egg-white-based diet with supplemental biotin. $ZnCO_3$ and all the other mineral supplements were reagent grade (J. T. Baker, Phillipsburg, NY, USA, and Pflatz and Bauer, Watersbury, CT, USA). The basal Zn-deficient diet closely resembled the AIN-93G diet for growing rats (Reeves *et al.* 1993) except that the mineral mix was reformulated to meet the requirements for P when egg-white is used as the source of protein (Reeves, 1996). The diet contained less than 0·6 mg Zn/kg as assessed by inductively coupled Ar plasma atomic emission spectrometry (ICAP-AES) (Nielsen *et al.* 1988). Standard reference materials (National Institute of Standards and Technology, Gaithersburg, MD, USA; no. 1572 citrus leaves and no. 1577a bovine liver) were used as quality control materials in the analysis.

Table 1. *Basal zinc-deficient diet (g/kg)**

Maize starch	427·495
Egg white (800 g protein/kg)	200·00
Dextrinized starch (900 g tetrasaccharides/kg)	100·00
Glucose	100·00
Soyabean oil	75·00
Fibre (Solka Floc)	50·00
Mineral mix (excluding Zn)†	35·00
Vitamin mix (AIN-93 modified)‡	10·00
Choline bitartrate	2·50
Tert-butylhydroquinone	0·005

* Contained < 0·6 mg Zn/kg diet as assessed by inductively coupled Ar plasma atomic emission spectrometry (Nielsen *et al.* 1988).

† Comprised (g/kg): $CaCO_3$ anhydrous 203·76, $CaHPO_4$ 332·27, KH_2PO_4 126·79, MgO 23·68, $Na_2SiO_3 . 9H_2O$ 7·25, $FeSO_4 . 7H_2O$ 4·98, $MnCO_3$ 0·63, $CuCO_3 . Cu(OH)_2$ 0·30, $KCr(SO_4)_2 . 12H_2O$ 0·275, H_3BO_3 0·0185, NaF powder 0·0635, $NiCO_3$ (45 % Ni) 0·0635, SnO 0·0162, NH_4VO_3 0·0132, $(NH_4)_6Mo_7O_{24} . 4H_2O$ 0·0106, Na_2SeO_4 anhydrous 0·01025, KIO_3 0·010, glucose to 1000 g.

‡ Comprised (g/kg): nicotinic acid 3·00, Ca pantothenate 1·00, pyridoxine HCl 0·70, thiamin HCl 0·60, riboflavin 0·60, pteroylglutamic acid 0·20, D-biotin 0·02, cyanocobalamin (1 g/kg in mannitol) 2·50, dl-α-tocopheryl acetate 15·00, retinyl palmitate 0·80, cholecalciferol 0·25, phylloquinone 75·0 mg, glucose to 1000 g.

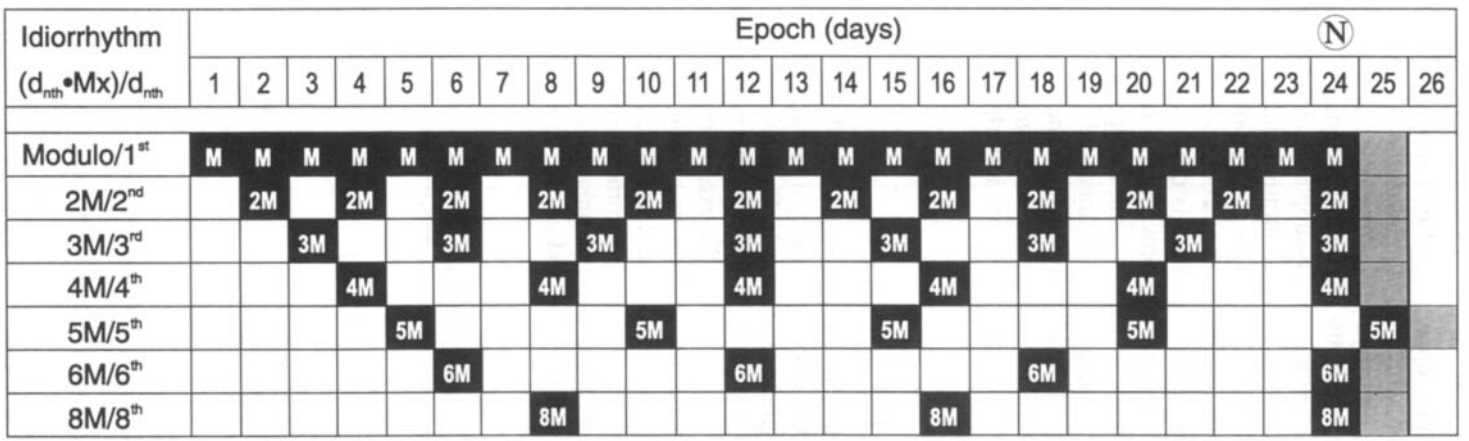

Fig. 1. Design of the idiorrhythmic feeding experiment. (□), No zinc; (■), zinc dosing day; (▨), no food. The entire 24 d experimental period is referred to as an epoch (E). The term idiorrhythm (I) refers to the zinc dose rate which is expressed as:

$$[d_{nth}(Mx)]/d_{nth} \text{ (mg Zn/kg per } d_{nth}),$$

where d_{nth} is the sequential number of zinc dosing day and Mx is the modulo (dose–time equivalent, expressed as mg Zn/kg per d or mg Zn/kg per E). Using this nomenclature, where x is 3: M3 = 3 mg Zn/kg per d or 72 mg Zn/kg per E and the associated I values are: 3/1, 6/2, 9/3, 12/4, 15/5, 18/6 and 24/8. Similarly, M6 = 6 mg Zn/kg per d or 144 mg Zn/kg per E, I = 6/1, 12/2, 18/3, 24/4, 30/5, 36/6 and 48/8; M12 = 12 mg Zn/kg per d or 288 mg Zn/kg per E, I = 12/1, 24/2, 36/3, 48/4, 60/5, 72/6 and 96/8; M24 = 24 mg Zn/kg per d or 576 mg Zn/kg per E, I = 24/1, 48/2, 72/3, 96/4, 120/5, 144/6 and 192/8. (Ⓝ), Nodus, the common denominator for all idiorrhythms, the span of E.

Four representative dose–time equivalent Zn modulos were chosen over a 24 d idiorrhythmic E. The four modulos were, Zn-deficient (M3; 3 mg Zn/kg per d_1), low Zn (M6; 6 mg Zn/kg per d_1), adequate Zn (M12; 12 mg Zn/kg per d_1), and ample Zn (M24; 24 mg Zn/kg per d_1). Each modulo had seven analogous dose-rate idiorrhythms arranged in an increasing order: (a) I = Mx/1; 3, 6, 12, or 24 mg Zn/kg per d_1 fed daily, (b) I = 2Mx/2; 6, 12, 24, or 48 mg Zn/kd per d_2 fed every other day and separated by a day of feeding a Zn-deficient diet, (c) I = 3Mx/3; 9, 18, 36, or 72 mg Zn/kg per d_3 fed every third day and separated by 2 d of feeding a Zn-deficient diet, (d) I = 4Mx/4; 12, 24, 48, or 96 mg Zn/kg per d_4 fed every fourth day and separated by 3 d of feeding a Zn-deficient diet, (e) I = 5Mx/5; 15, 30, 60, or 120 mg Zn/kg per d_5 fed every fifth day and separated by 4 d of feeding a Zn-deficient diet, (f) I = 6Mx/6; 18, 36, 72, or 144 mg Zn/kg per d_6 fed every sixth day and separated by 5 d of feeding a Zn-deficient diet, and (g) I = 8Mx/8; 24, 48, 96, or 192 mg Zn/kg per d_8 fed every eighth day and separated by 7 d of feeding a Zn-deficient diet. Each Zn dose–time equivalent modulo comprised seven analogous Zn dose-rate idiorrhythms and was run as an independent experiment. The expected *v.* analysed Zn contents of all twenty-eight experimental diets are shown in Table 2.

Separate groups of rats underwent the conventional dose–response treatment where graded amounts of dietary Zn were fed continuously (every day, I = Mx/1) and over the same length of time as idiorrhythmically-fed animals. The groups of animals continuously fed with various doses of Zn were randomly distributed and often replicated across all four modulos to control the chance for fluctuation relative to the fact that modulo groups were investigated separately (See Appendix). Consequently, the data for continuously-fed animals across all four modulos are shown together in the corresponding tables and figures as if they were coming from a single experiment. The mean expected (analysed) Zn contents of these diets were (mg Zn/kg): 3 (3·1), 6 (6·4), 12 (11·8), 24 (19·5), 60 (58·0), 120 (104), 240 (208), 300 (277), and 400 (415). The National Research Council (1995) Zn requirement of 12 mg Zn/kg diet for growing rats is equal to that of the Zn dose-rate I = 12/1 (12 mg Zn/kg per d_1). Therefore, $I_{M12} = 12/1$ was considered the control idiorrhythm.

Table 2. *Zinc concentration (mg Zn/kg diet) of experimental diets fed idiorrhythmically*

(Expected (Ex) *v.* analysed (An) values are means and standard deviations for three determinations)

	Modulo* (mg Zn/kg per d_1)											
	M3 Zn deficient			M6 Low Zn			M12 Adequate Zn			M24 Ample Zn		
	Ex	An		Ex	An		Ex	An		Ex	An	
Idiorrhythm* (mg Zn/kg per d_{nth})		Mean	SD		Mean	SD		Mean	SD		Mean	SD
0	0	0·5	0·03	0	0·5	0·03	0	0·5	0·04	0	0·6	0·07
M/1	3	2·8	0·02	6	6·4	0·3	12	11·2	0·1	24	19·5	0·2
2M/2	6	5·8	0·2	12	13·7	0·4	24	19·5	0·2	48	40·7	0·5
3M/3	9	8·6	0·2	18	19·0	0·3	36	30·6	0·5	72	65·4	0·5
4M/4	12	10·8	0·1	24	24·5	0·3	48	40·7	0·5	96	83·4	0·3
5M/5	15	13·7	0·2	30	29·6	2·1	60	52·8	0·6	120	104·4	7·8
6M/6	18	16·9	0·4	36	37·7	0·2	72	65·4	0·5	144	135·9	3·6
8M/8	24	21·8	0·2	48	50·3	0·4	96	83·4	0·3	192	168·1	2·0

* For details of terms and procedures, see Fig. 1 and pp. 174–178.

Experimental animals

This study was approved by the Animal Use Committee of the U.S. Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center and was in accordance with the guidelines of the National Research Council (1985) on the experimental use of laboratory animals.

Weanling, 21-d-old, male Sprague–Dawley rats (Sasco, Omaha, NE, USA) were kept in individual stainless steel cages with wire-mesh floors and located in a temperature- (22–24°) and humidity- (44–55 %) controlled room on a 12:12 h dark–light cycle. They were given free access to powdered experimental diets and deionized water (Super Q System, Millipore Corp., Bedford, MA, USA). After being fed on their respective diets for 24 d, and deprived of food overnight (16 h), most of the rats were killed by halothane inhalation. Some of the rats were subjected to restraint stress by enclosing them individually in a plastic animal holder and allowing them to remain there for 30 min. This restricted the animal's movement but did not completely immobilize it (Klevay & Halas, 1991). These rats were then killed by decapitation and the expression of aortal HSP70mRNA was determined.

Immediately after the rats were killed, the aortas and small intestines were dissected out and the mucosal lining scraped from the intestine. Both aortas and mucosal scrapings were kept frozen at − 80° until analysed for HSP70mRNA and iMT respectively. Zn content was determined in the dry ash of the left femur and left upper incisor (Momčilović *et al.* 1975*a,b*) by ICAP-AES.

Intestinal metallothionein by the cadmium–haemoglobin displacement assay

Small-intestinal mucosa was analysed in idiorrhythmic M12, M24, and all groups of conventionally-fed rats. The mucosa was homogenized and prepared for iMT analysis by following the method of Eaton & Toal (1982), except that a portion of the homogenate was heated for 5 min at 95° and centrifuged for 5 min at 10 000 ***g***. The Tris buffer also contained 1 mmol 2-mercaptoethanol/l, pH 7·4. The amount of iMT in the mucosa was expressed as Cd-binding potential and calculated based on the assumption that 7·0 g-atoms of Cd are bound per mole of iMT and that the molecular mass of Cd-iMT is about 6500 Da (Reeves, 1995). Values were expressed as a percentage of the average value of iMT obtained from rats fed on the control idiorrhythm, $I_{M12} = 12/1$.

Heat shock protein 70 mRNA isolation and Northern hybridization analysis

The restraint-stress-induced aortal HSP70mRNA was measured in selected groups of animals. The aorta was homogenized at high speed for 10 s (Tekmar, Cincinnati, OH, USA) in RNAzol (Tel-Test, Inc., Friendwood, TX, USA). Total cellular RNA was isolated by using the manufacturer's protocol (Tel-Test, Inc.) and was quantified spectrophotometrically before loading samples onto agarose gels. For Northern analyses, 10 μg total RNA was denatured in formaldehyde and fractioned in 10 g/l agarose gel containing 2·2 mol formaldehyde/l. RNA was transferred to Genescreen Plus membranes (DuPont, Boston, MA, USA) according to the manufacturer's recommended conditions in 1·5 mol NaCl and 0·15 mol of sodium citrate/l, then baked for 2 h at 80°; membranes were hybridized and washed at 65° and the blot was probed with a HSP70 complementary DNA (cDNA).

Probes and labelling reactions

A cDNA for HSP70, isolated from a Chinese hamster ovary cell line, was provided by Dr Albert J. Fornace, Jr (National Cancer Institute, Bethesda, MD, USA) (Fornace *et al.* 1989). This probe recognizes both constitutive and inducible members of the HSP70 gene family (Church & Gilbert, 1984; Blake *et al.* 1990). Purified inserts were labelled with ^{32}P-2′-deoxycytidine 5′-triphosphate (DuPont–NEN, Boston, MA, USA) by using the random primer method of Feinberg & Vogelstein (1983).

Densitometric analysis of Northern blots

Before scanning the autoradiograms, a photographic negative of the ethidium bromide-(Sigma, St. Louis, MO, USA) stained samples was used to eliminate degraded or unevenly loaded samples. Initially, if any sample appeared degraded, as evidenced by a less than 2:1 ratio of 18S:28S ribosomal RNA(rRNA) or a noticeable smearing, it was removed from the analysis. The negative of the ethidium-stained sample was scanned densitometrically along with the resultant autoradiogram. If the density of the 18S or 28S rRNA bands of a given sample deviated by more than 10 % from the mean density for all samples in a gel then it too was removed from the analysis. Densitometric values were then determined from digitized images of autoradiograms (Imaging Research Inc., St. Catharines, Ontario, Canada). Values were corrected for background and expressed as a percentage of the average signal in rats fed on the control $I_{M12} = 12/1$ (control idiorrhythm HSP70mRNA = 1·00). Gels were always prepared so that samples from all treatment groups were present on a single gel.

Statistical analysis

Results are expressed as means and standard deviations. All differences were considered significant if $P \leq 0{\cdot}05$. The effect of four different dietary Zn dose–time equivalent modulos, each with seven analogous Zn dose-rate idiorrhythms, on aortal HSP70mRNA, and Zn content of the femur and the incisor were assessed by ANOVA. The Ryan–Einot–Gabriel–Welsh (REGW) multiple *F* test (Einot & Gabriel, 1975) was used to determine if the mean values between idiorrhythms of the same modulo were significantly different. The iMT results were analysed with a two-way ANOVA, differences between different modulos and analogous idiorrhythms were assessed by the REGW multiple *F* test, and the differences between idiorrhythms were assessed with Tukey's studentized range test (Kleinbaum & Kupper, 1978). The data for intestinal iMT and aortal HSP70mRNA were normalized against the control idiorrhythm before statistical analysis to control for the between-experiment variability.

The data for iMT induction in rats fed continuously with graded amounts of Zn in a conventional dose–response regimen (I = Mx/l) were normalized to the mean value of iMT in the control idiorrhythm and fitted to a ln-ln regression model (Kleinbaum & Kupper, 1978). The respective comparative data for total femur and incisor Zn for the same rats were fitted to a break-point assay model as described by Hunt & Johnson (1992). The fitted models served as reference calibration standards for assessing the biological effects of idiorrhythmic Zn dose-rate feeding.

RESULTS

Presentation of the data follows the dynamics of the natural physiological sequence: (a) entry of Zn into the gastrointestinal mucosa where iMT is induced, (b) circulatory distribution to the aorta where HSP70mRNA expression occurs, and (c) accumulation of Zn in the femur and incisor (amount and concentration). They are shown separately for the conventional dose–response and idiorrhythmic dose-rate feeding model respectively.

Intestinal metallothionein

For the conventional dose–response model the induction of iMT by continuous daily feeding of graded amounts of dietary Zn was fitted to a ln-ln regression model (Fig. 2). The ln(iMT) rose linearly with ln(dietary Zn) over the range from 3 to 415 mg Zn/kg per d_1.

For the idiorrhythmic dose-rate model the effect of Zn dose-rate idiorrhythm and Zn dose–time equivalent modulo on the relative concentration of iMT is shown in Table 3. Overall, the relative concentration of iMT was the same for rats fed on Zn dose–time equivalent M12 (adequate Zn) or M24 (ample Zn) ($P = 0{\cdot}69$). However, there was an overall effect of idiorrhythm on iMT, because rats fed by $I_{M12} = 24$ and $I_{M24} = 48$ mg Zn/kg per d_2 had significantly higher iMT than rats fed by the other idiorrhythms ($P < 0{\cdot}01$). The analysis also showed that iMT induction in response to Zn within the

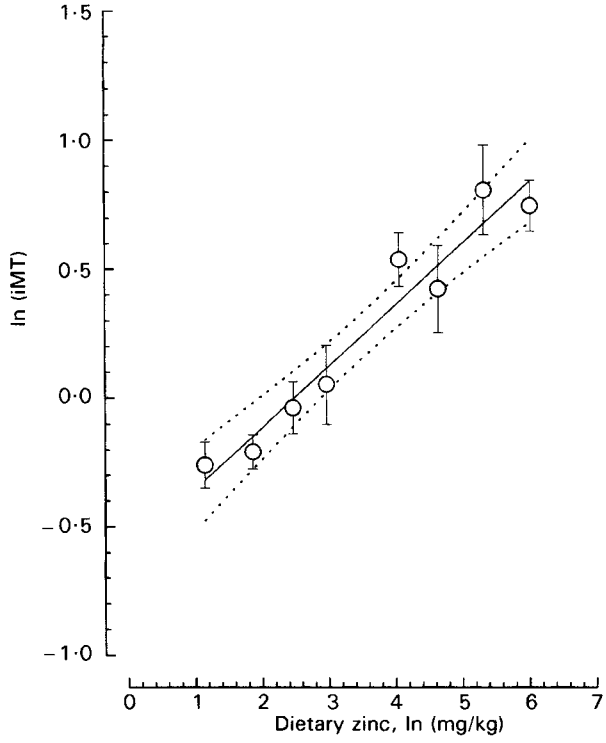

Fig. 2. Regression analysis of data relating ln intestinal metallothionein (ln(iMT)) concentrations with continuous daily feeding of graded amounts of dietary zinc over an entire idiorrhythmic epoch. Values are means with standard deviations represented by vertical bars for eight animals. (---), 95% confidence interval. Control idiorrhythm (iMT for $I = 12/1) = 1{\cdot}0$. For details of terms and procedures, see Fig. 1 and pp. 174–178. The equation for the curve is: $\ln(y) = -0{\cdot}59 + 0{\cdot}24\ln(x)$, r^2 0·942.

Table 3. *Effect of zinc dose-rate idiorrhythm (I) and zinc dose–time equivalent modulo (M) on induction of intestinal metallothionein (iMT; expressed as a percentage of control iMT) after a 24 d idiorrhythmic epoch**

(Mean values and standard deviations for eight rats)

Idiorrhythm* (mg Zn/kg per d_{nth})	Modulo* (mg Zn/kg per d_1)					
	I	M12[A]		I	M24[A]	
		Mean	SD		Mean	SD
M/1[B]	12/1†	1·00[bcd]	0·26	24/1	1·16[bcd]	0·47
2M/2[A]	24/2	1·87[a]	0·51	48/2	1·61[a]	0·43
3M/3[B]	36/3	0·84[cd]	0·26	72/3	1·03[bcd]	0·12
4M/4[B]	48/4	1·17[abc]	0·32	96/4	1·15[bcd]	0·30
5M/5[B]	60/5	1·48[ab]	0·41	120/5	0·49[d]	0·16
6M/6[B]	72/6	1·08[bcd]	0·30	144/6	1·40[ab]	0·80
8M/8[B]	96/8	0·85[bcd]	0·18	192/8	1·24[ab]	0·51

[A, B] Two way ANOVA: M, $P < 0·6878$; I, $P < 0·0001$; M × I interaction, $P < 0·0001$. M or I values bearing different upper case superscripts were significantly different ($P < 0·05$) by the REGW multiple F test (Einot & Gabriel, 1975).

[a,b,c,d] Mean values not sharing a common lower case superscript letter were significantly different, $P < 0·05$ (Tukey's studentized range (HSD) test).

* For details of terms and procedures, see Fig. 1 and pp. 174–178.

† Control idiorrhythm.

modulo was bi-modal, with a secondary peak at $I_{M12} = 60$ mg Zn/kg per d_5 and $I_{M24} = 144$ mg Zn/kg per d_6. The secondary peak shifted towards the higher idiorrhythms with an increase in the Zn dose–time equivalent modulo from M12 to M24. The lowest relative concentration of iMT was 0·84 when rats were fed by $I_{M12} = 36$ mg Zn/kg per d_3, and 0·49 when they were fed by $I_{M24} = 120$ mg Zn/kg per d_5.

The relative concentration of iMT in rats fed on the idiorrhythmic diets was always lower than that of rats continuously fed on diets containing 60 mg Zn/kg per d_1 or more. It was approximately equal to that in rats fed by $I_{M12} = 24/2$ and $I_{M24} = 48/2$, which apparently had the greatest iMT induction capacity.

Restraint-stress-induced heat-shock protein 70 mRNA expression in the aorta

For conventional dose–response feeding an initial experiment (Momčilović *et al.* 1995) showed that values (expressed as a percentage of the control idiorrhythm) for aortal HSP70mRNA expression of restrained rats (n 6) fed with 3, 6, 12, and 300 mg Zn/kg per d_1 daily were 0·52 (SD 0·27), 1·27 (SD 0·30), 1·00 (SD 0·55), and 1·03 (SD 0·55) respectively (3 *v.* 6 mg Zn/kg per d_1, $P < 0·05$; Tukey contrasts). Aortal HSP70mRNA expression was lowest in rats fed continuously with the lowest concentration of dietary Zn ($I = 3$ mg Zn/kg per d_1). Increasing dietary Zn to 300 mg Zn/kg per d_1, well above that of the control idiorrhythm, did not affect the expression of aortal HSP70mRNA induced by restraint stress.

For the idiorrhythmic dose-rate model the effects of Zn dose-rate idiorrhythm and Zn dose–time equivalent modulo on aortal HSP70mRNA expression are shown in Table 4. It should be noted that the total number of samples analysed was small; however, none of the samples had to be excluded because of technical reasons. Rats that were fed continuously with 3 mg Zn/kg per d_1 had decreased HSP70mRNA expression compared with rats fed by the control idiorrhythm ($P < 0·05$; Fig. 3). The aortal restraint-stress-induced

Table 4. *Effect of zinc dose-rate idiorrhythm and zinc dose–time equivalent modulo (M) on the expression of restraint stress-induced aortal heat-shock protein 70mRNA (expressed as a percentage of control†) after a 24 d idiorrhythmic epoch**

(Mean values and standard deviations for four rats)

Idiorrhythm* (mg Zn/kg per d_{nth})	Modulo* (mg Zn/kg per d_1)					
	M3		M12		M24	
	Mean	SD	Mean	SD	Mean	SD
M/1	$0{\cdot}36^{c}$	0·43	$1{\cdot}00^{a}$	0·23	-	
3M/3	-		$0{\cdot}88^{abc}$	0·33	$0{\cdot}84^{abc}$	0·25
5M/5	-		$0{\cdot}51^{abc}$	0·26	$0{\cdot}94^{ab}$	0·21
8M/8	-		$0{\cdot}84^{abc}$	0·20	$0{\cdot}37^{bc}$	0·36

^{a,b,c} Mean values not sharing a common superscript letter were significantly different ($P < 0{\cdot}05$) by the REGW multiple F test (Einot & Gabriel, 1975).

* For details of terms and procedures, see Fig. 1 and pp. 174–178.

† Control idiorrhythm = 12/1.

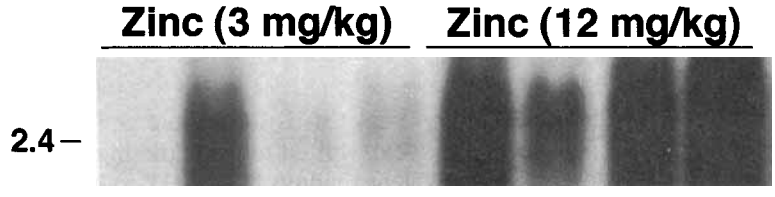

Fig. 3. Restraint-stress-induced heat-shock protein (HSP) 70 mRNA in the aorta of rats fed with 3 mg Zn/kg per d_1 (idiorrhythm 3/1) or 12 mg Zn/kg per d_1 (idiorrhythm 12/1). Total RNA was isolated from the aorta of individual animals and assayed by Northern blot analysis. The HSP70 expression shown is for 10 μg total RNA from the aortas of four rats for each dietary group. The blot was probed with a HSP70 complement DNA probe to recognize both constitutive and inducible members of the HSP70 gene family. Molecular mass marker (2·4 kb) is indicated on the left.

HSP70mRNA expression was lower for all of the tested idiorrhythms when compared with the control idiorrhythm. However, the differences were statistically significant only if rats were fed by the high degree $I_{M24} = 192$ mg Zn/kg per d_8 ($P < 0{\cdot}05$).

Femur and incisor

For the conventional dose–response model the relationship between Zn deposition in the femur and incisor after conventional daily feeding of graded doses of dietary Zn is shown in Fig. 4. The break-point assay used here reasonably described the observed dose–response pattern of Zn deposition in the femur and incisor over the entire range of dietary Zn concentrations tested (3–415 mg Zn/kg per d_1). The values for the break points that divided the initial rapid response phase of Zn deposition from the latter, almost flat, phase were 13·7 mg Zn/kg per d_1 and 14·4 mg Zn/kg per d_1 for the femur and incisor, respectively. It is of interest that these values closely match the dietary Zn requirement for the growing rat established by the U.S. National Research Council (1995). The difference in Zn requirements between the two calcified tissues did not exceed 5 %, a remarkably close correspondence for two sets of independent indicators.

For the idiorrhythmic dose-rate model the effects of Zn dose-rate idiorrhythm and Zn dose–time equivalent modulo level on femur and incisor Zn amount and concentration are

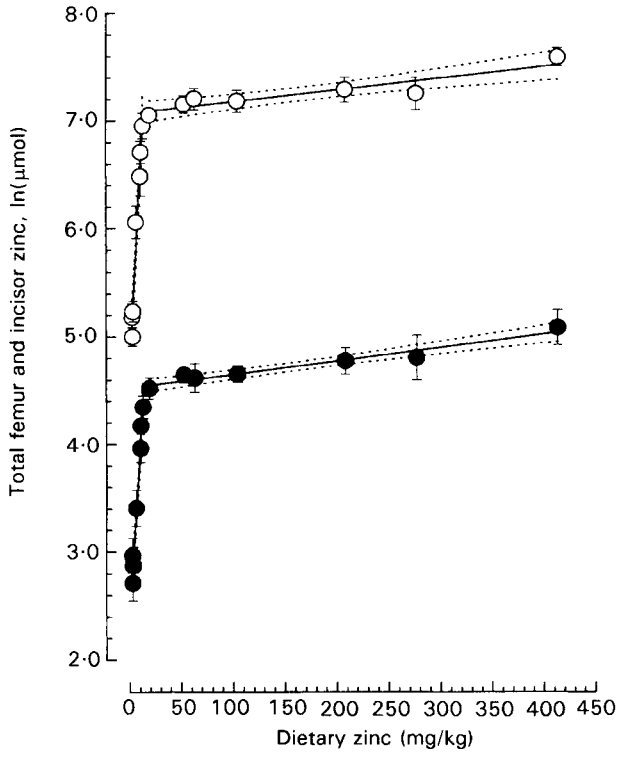

Fig. 4. Break-point analysis of the data relating femur (○) and incisor (●) zinc with continuous daily feeding of graded amounts of dietary zinc over an entire idiorrhythmic epoch. Values are means for eight animals with standard deviations represented by vertical bars; (---), 95 % confidence interval. The equations for the two portions of the femur curve are $\ln(y) = 4{\cdot}63 + 0{\cdot}18x$ when dietary zinc is less than 13·7 mg/kg, and $\ln(y) = 7{\cdot}07 + 0{\cdot}001x$ when dietary zinc is greater than 13·7 mg/kg. For the two portions of the incisor curve, the equations are $\ln(y) = 2{\cdot}39 + 0{\cdot}15x$ when dietary zinc is less than 14·4 mg/kg, and $\ln(y) = 4{\cdot}53 + 0{\cdot}001x$ when dietary zinc is greater than 14·4 mg/kg.

shown in Fig. 5 and Fig. 6 respectively. Among rats fed on the Zn-deficient M3 the amount and concentration of Zn in femur and incisor gradually increased with the progression of the idiorrhythms. Femur Zn content and femur and incisor Zn concentrations were lowest for I = 3 mg Zn/kg per d_1, and highest for I = 24 mg Zn/kg per d_8 ($P < 0{\cdot}05$ for the femur and incisor pairs). When Zn dose–time equivalent was the Zn-deficient M3 then the larger amplitude of Zn dose-rate resulted in a greater deposition of Zn in the femur and incisor, despite a low dose-rate frequency.

At the low Zn M6 the progression of the idiorrhythmic series resulted in a complex, bi-modal pattern of Zn deposition in the femur. Femur Zn content and concentration decreased from I = 6 mg Zn/kg per d_1, to I = 12 mg Zn/kg per d_2 and reached their nadir at I = 18 mg Zn/kg per d_3, and I = 24 mg Zn/kg per d_4 respectively. Further progression of the idiorrhythmic series to I = 30 mg Zn/kg per d_5 resulted in a significant ($P < 0{\cdot}05$) rise in femur Zn content and concentration. This secondary increase peaked at I = 30 mg Zn/kg per d_5 and I = 36 mg Zn/kg per d_6. The end result was that the animals fed daily by the continuous I = 6/1 had the same Zn concentration in the femur as did the rats fed by I = 30/5 and I = 36/6 ($P < 0{\cdot}05$). A similar but less pronounced pattern of Zn deposition occurred for Zn content and concentration in the incisor.

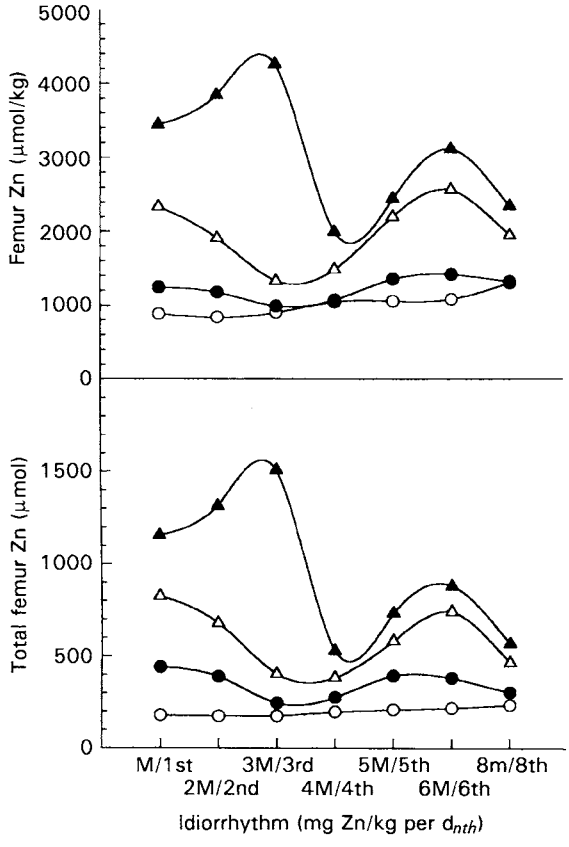

Fig. 5. The impact of zinc dose-rate idiorrhythm and zinc dose–time equivalent modulo (M) on zinc deposition in the femur of weanling male rats. Values are means for eight rats. (○), M3; (●), M6; (△), M12; (▲), M24. The root mean square error (an estimate of overall standard deviation) is equal to 300 for femur zinc concentration and 80 for total femur zinc. For details of terms and procedures, see Fig. 1 and pp. 174–178.

The series of adequate Zn M12 idiorrhythms generated the same non-linear, bi-modal pattern of Zn deposition in the femur and incisor as that of the preceding low Zn M6 idiorrhythmic series, although of greater magnitude. Both femur and incisor Zn contents and concentrations decreased gradually from the initial, continuous daily I = 12 mg Zn/kg diet per d_1, to reach the lowest level at I = 48 mg Zn/kg per d_4. Thereafter, the values rose for I = 60 mg Zn/kg per d_5, and reached their secondary peak at I = 72 mg Zn/kg per d_6. Femur and incisor Zn contents and concentrations at I = 72/6 reached the same level as in rats fed continuously by the control I = 12/1. All M12 idiorrhythms had greater femur and incisor Zn contents and concentrations than the analogous M6 idiorrhythms.

When dose–time equivalent was increased two times above the daily Zn requirement (ample Zn M24) it resulted in yet another response pattern of Zn accumulation in the femur and incisor. Their contents and concentrations first increased as the idiorrhythmic series progressed from I = 24 mg Zn/kg per d_2, to peak at I = 72 mg Zn/kg per d_3, then dropped about 50 % when I = 96 mg Zn/kg per d_4. However, when that drop was compared with the adequate Zn M12 idiorrhythms the values for $I_{M24} = 96/4$ were similar to that for $I_{M12} = 12/1$ idiorrhythm; the rats receiving the ample Zn M24 retained more Zn in the

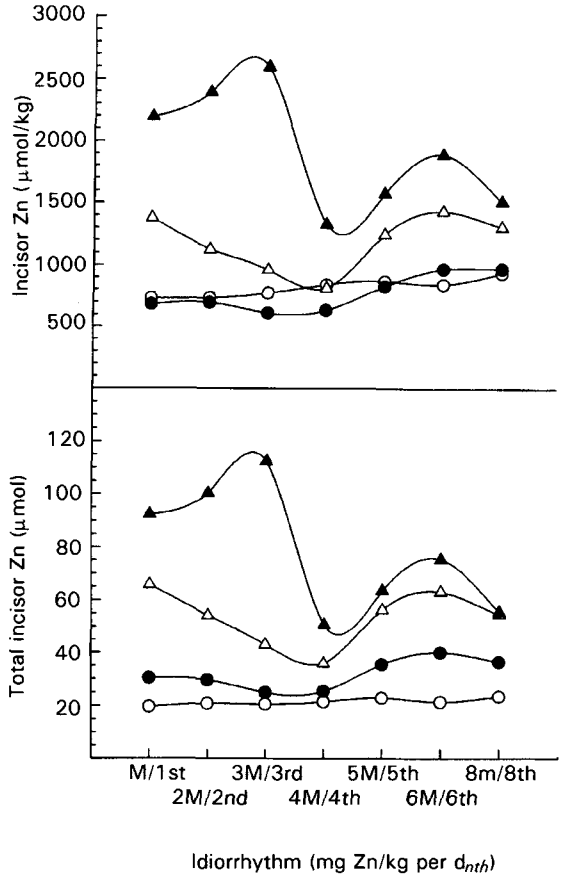

Fig. 6. The impact of zinc dose-rate idiorrhythm and zinc dose–time equivalent modulo (M) on zinc deposition in the incisor of weanling male rats. Values are means for eight rats. (○), M3; (●), M6; (△), M12; (▲), M24. The root mean square error (an estimate of overall standard deviation) is equal to 182 for incisor zinc concentration and 8 for total incisor zinc. For details of terms and procedures, see Fig. 1 and pp. 174–178.

femur and incisor than rats receiving the Zn-adequate M12. The secondary peak occurred when Zn was dosed every sixth day, I = 144 mg Zn/kg per d_6, as was already observed with the analogous $I_{M6} \simeq 36/6$ and $I_{M12} = 72/6$. The I = 144/6 barely matched that of the continuous I = 24/1 regarding Zn accumulation in the femur and incisor, and was significantly lower than that for I = 48/2 and I = 72/3 ($P < 0{\cdot}05$ for the femur and incisor).

DISCUSSION

The results of the present experiment show that the idiorrhythmic dose-rate variability of dietary Zn intake generates different patterns of Zn accumulation in the femur and incisor, iMT induction, and in the expression of aortal HSP70mRNA than the conventional dose–response feeding of a constant daily dose of dietary Zn. Evidently, different dose-rates of dietary Zn modify the biological response of experimental animals in a hitherto

unrecognized way indicating the important role that temporal variability in dietary Zn intake has on the functional metabolic response.

In contrast to the conventional daily feeding of graded amounts of dietary Zn in a classical dose–response model, the idiorrhythmic short time cycling of an excessive Zn load followed by a more protracted period of feeding the diet without Zn did not affect iMT and HSP70mRNA. Idiorrhythmic feeding resulted in an increase of iMT only when a double dose of Zn was fed every other day. Because iMT induction is greatest 18 h after gastric intubation (Menard *et al.* 1981), and because the half-life for iMT is considered to be less than 24 h (Richards & Cousins, 1976), the increase in iMT of rats fed with a double dose of Zn every other day ($I = 2M/2$) may reflect the accumulation of newly induced iMT on the yet unmetabolized iMT from the preceding Zn dosing period. The other idiorrhythms did not appreciably change iMT values above those of the control idiorrhythm. This suggests that the dosing days were separated by Zn-deficient periods long enough to preclude an accumulation of iMT, or any other tachyphylactic adaptation (Gershengorn, 1994). (Tachyphylaxis is a decreased biological response to a stimulus of constant strength, notably an excessive or pharmacological one.)

The final Zn dose in the conventional dose–response feeding of constant amounts of dietary Zn is considered to be the most important determinant of iMT concentration, but recent investigation showed that such induction of iMT with high doses of dietary Zn is not permanent. First it rises but thereafter it decreases to almost normal values over a prolonged period of feeding high Zn. This suggests that iMT induction may be regarded only as a temporary safety mechanism that impedes excessive Zn accumulation (Reeves, 1995). Apparently, idiorrhythmic dose-rate cycling of the dietary Zn load does not pose a biochemical risk to the organism that needs to be counterbalanced by an increase in iMT induction. The observation that iMT induction was not Zn dose-dependent under the conditions of idiorrhythmic feeding opens a possibility for bypassing the so called 'mucosal block' by adequate spacing of nutrient intake.

The present experiment with young growing rats confirmed the previous finding with young adult rats (Momčilović *et al.* 1993) that the expression of aortal HSP70mRNA induced by restraint stress is markedly decreased if rats are fed continuously on a Zn-deficient diet ($I_{M3} = 3/1$). Similarly idiorrhythmic feeding did not increase aortal HSP70mRNA expression in restrained rats. In fact, aortal HSP70mRNA expression for the ample Zn intake $I_{M24} = 192/8$ was significantly less than in the control group. Such a high idiorrhythmic bolus of dietary Zn is still below the dose that may adversely affect humans (Fosmire, 1990), and apparently does not act as a stress factor that would stimulate aortal HSP70mRNA expression.

Our results with the break-point assay of Zn bioavailability are in good agreement with those of Hunt & Jackson (1992). We inferred that the results of idiorrhythmic feeding could not have been influenced by the timing of each study because the data from a break-point assay on femur and incisor of continuously-fed animals came from random and replicated treatments across all four modules. Femur and incisor data showed a remarkably close correspondence with not more than 5 % difference between the two independent indicators. The existence of the break point is a clear demonstration that the deposition of Zn in the femur and incisor is controlled by an ultra-sensitive threshold switching mechanism. Ultra-sensitive is defined as a response in a biological system that is more sensitive than is to be expected from the classical hyperbola of Michaelis–Menton kinetics (Koshland, 1987). Small changes in the doses of dietary Zn below that indicated by the break point cause a very large change in output. The results fully correspond with the slope–ratio assay for the

assessment of Zn bioavailability from food as proposed by Momčilović *et al.* (1975*a*, 1976). Because of the operation of an ultra-sensitive threshold switch, an increase in dietary Zn above that indicated by the break point results in only a gradual deposition of Zn in the femur and incisor.

While the increase in the dose–time equivalent from adequate Zn M12 to ample Zn M24 resulted in a marked increase in Zn content and concentration of femurs and incisors for each of the analogous idiorrhythms, the iMT values, for the most part, remained unchanged. This indicates that significant amounts of dietary Zn were indeed absorbed without appreciable iMT induction. The magnitude of idiorrhythmically induced temporal increases in the deposition of Zn in femurs and incisors may be, in fact, much greater than the overall results show.

Deposition of minerals into bone is characterized by at least two phases, initial deposition in the so-called exchangeable or partially reversible fraction of the bone, and irreversible deposition in the bone crystal lattice (Marshall *et al.* 1973). The process occurs as a laminar deposition in the newly formed or intensively remodelled regions of the bone (Turner *et al.* 1993; Martin, 1994). The process is somewhat different in teeth; the rat incisors are growing by a undirectional and irreversible accretion of minerals (Bronner, 1964). As the number of days between dosing of Zn increased, the layers of Zn deposited in the bone would be separated by large layers of newly formed bone without Zn. However, a mere Zn analysis of the femur and incisor would not detect whether the calcified tissues were uniformly labelled or not; therefore, the high amount of bone Zn laid down idiorrhythmically would be obscured within the total bone mass.

The results on femur and incisor Zn accumulation showed that, although the offered dose was the same for all the idiorrhythmically-fed animals that belonged to the same modulo, Zn deposition differed, i.e. the Zn dose-rate idiorrhythm changed the metabolic efficiency of Zn. We did not measure food intake, but be that as it may, any change in food intake, appetite, palatability, or metabolic utilization, either separately or in combination, results from a change in the Zn dose-rate idiorrhythm. In other words, a change in Zn deposition in the indicator tissues, femur and incisor, is the common vector of all changes that may occur in the different components of the whole animal.

The idiorrhythmic feeding regimen revealed that weanling rats have a different response pattern of Zn deposition in the femur and incisor depending on whether the total dietary Zn supply is deficient, low, adequate, or ample. It is tempting to conclude that if partitioning of a totally available amount of Zn in an otherwise Zn-scarce nutritional environment can be controlled, it may be advantageous to indulge in Zn excess from time to time rather than to consume a moderately Zn-deficient diet all the time. Indeed, such a behavioural phenomenon has been observed in all the known human cultures where long stretches of simple and often inadequate diets are regularly interrupted by seemingly irrational carnival feasts.

Our results clearly demonstrate that, in contrast to the classical dose–response model, the component of time should not be ignored in the analyses of biological systems. The idiorrhythmic dose-rate feeding offers the possibility of bridging the gap between the classical dose–response studies and time-related phenomena of chronobiology. A large number of infradian rhythms (> 24 h) have been observed in weanling rats, one of which has a duration of 5·4 d (Mercer *et al.* 1993). This corresponds to the secondary peak of Zn deposition in the femur and incisor in the present experiment, and that of body growth as observed in a previous study (Momčilović, 1995). This may be interpreted as an interaction

between the extrinsic idiorrhythm of nutrient intake and an underlying intrinsic infradian rhythm in the body. We think that the biochemical basis for such a biochemical inference may be related to metabolic channelling (Ovadi, 1991), futile metabolic cycles (Belfiore & Iannello, 1990) and conditions when certain biological functions are controlled by two or more subsystems that may differ in their response time (Hurwitz *et al.* 1987).

Homeostasis and biological rhythms are both well-known natural phenomena but only rarely have investigators connected the two (Hyndman, 1974). Like any other physiological signal that elicits a response (Sato, 1971), nutrient intake acts as a time signal for circadian rhythms (Johnson, 1992), so that in *ultima linea*, the whole gastrointestinal tract can be viewed as a specific sensory organ that processes the nutritive signal. Most of the existing literature related to bioperiodicity aims (a) to prove that biological rhythms exist in and of themselves, and that they are not induced or entrained by light or food or some other signal, and (b) to separate circadian and learned rhythms on the basis of differences in pacemaker states. In contrast, idiorrhythmic feeding offers an active experimental approach that can unmask the underlying bioperiodicity of the organism, which is viewed as an integrated gestalt phase space (Momčilović, 1995). (Gestalt phase space is defined as a structure or configuration of physical, biological or psychological phenomena integrated to constitute a functional unit with properties non-derivable from its parts.) Indeed, we think that nutrition and metabolism should be viewed more as a metabolic network analogous to the neural network, if we are ever to come close to a clearer understanding of adaptability, addiction, tolerance, and/or the impact of change in metabolic fuels.

The possible implications of the idiorrhythmic merging of dose with time, and beyond the conventional dose–response model, may be far reaching. It was argued that with more information about nutrient–gene interactions and individual genetic identity, it may be possible to genotype populations so that food recommendations can be made on a more rational and individualistic basis; one that includes not only the dimensions of sex, age, and body size, but also the dimension of the genetic code so that we may choose foods that potentiate our genes for good health (Berdanier, 1993). The results of studies using idiorrhythmic dose-rate variability suggest that the dimension of time stimulation or nutrient signalling, and their metabolic networking within the biological gestalt phase space, should be considered essential for meaningful promotion of the nutritional genes. Otherwise, as D'Arcy Thompson said, 'Sooner or later, nature does everything that is physically possible. Our problem is, that which is physically possible may not be good enough' (cited by Waterlow, 1986).

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APPENDIX

Experimental plan

Modulo...	M3	M6	M12*	M24*
Start date...	17/7/92	9/6/93	23/9/93	30/9/93
Finish date...	11–12/8/92	5–6/7/93	18–19/10/93	25–26/10/93
Animal no.		Idiorrhythm†		
1–8	3/1	6/1	12/1	24/1§
9–16	6/2	12/2‡	24/2§	48/2§
17–24	9/3	18/3	36/3	72/3§
25–32	12/4‡	24/4	48/4§	96/4§
33–40	12/1‡	30/5	60/1‡	120/1‖
41–48	15/5	36/6	60/5‡	120/5‖
49–56	18/6	48/8	72/6§	144/6
57–64	24/8	3/1	96/8§	192/8
65–72	300/1	12/1‡	120/1‖	240/1
73–80		60/1		3/1
81–88		400/1		

* The start dates of M12 and M24 were only 1 week apart, i.e. they overlapped.
† Idiorrhythms of the format x/1 represent continuous feeding.
‡ Identical diet was fed either continuously or idiorrhythmically within the same modulo.
§ Identical diets were fed idiorrhythmically between the different modulos.
‖ Identical diet was fed both idiorrhythmically and continuously within and between the modulos.

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