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# Quantitative assessment of the effects of variability in dietary zinc dose-rate idiorrhythms upon zinc deposition in bone of weanling rats by using a slope-ratio assay

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*We quantitatively assessed the effect of the idiorrhythmic dose-rate variability in dietary zinc intake on zinc deposition in the femur and incisor of weanling rats by the slope-ratio analysis of analogous idiorrhythms. In idiorrhythmic feeding the dose (x) is viewed over the period of the entire experimental epoch as a dose-time equivalent modulo (Mx) that can be divided into a series of equal products of different doses with different frequency (idiorrhythm, I). Each I is administered in a regularly recurring pattern to deliver the same dose as that from a standard daily regimen of zinc, albeit at a different rate;* $Ix = [d_{nth}(Mx)]/d_{nth}$, *where* $d_{nth}$ *is the sequential number of zinc dosing day separated by 1 to 7 days of feeding the diet without zinc when* $d_{nth} > 1$. *Four different Mx were tested, M3, M6, M12, and M24 providing 3, 6, 12, and 24 mg* $Zn \cdot kg^{-1}$ *diet* $\cdot d^{-1}$, *respectively, over a 24 day epoch; each Mx had seven analogous Ix of different dose sizes, but the same dosing frequency (*$d_{nth}$*). The slopes for the M3, M6, and M12 analogous idiorrhythms showed that zinc deposition in the femur and incisor, as a measure of metabolic availability, varied considerably with spacing of the dose with time. Metabolic availability of zinc progressively decreased by 50% from* I = Mx/*1 to* I = 4Mx/*4. Then it rose to approach the initial* I = Mx/*1 values of animals fed zinc daily for* I = 5Mx/*5 and* I = 6Mx/*6, before it finally dropped again to the bottom level for* I = 8Mx/*8. The results showed that the impact of zinc dose-rate in dietary zinc intake is an important determinant of an adequate supply of metabolic zinc.* (J. Nutr. Biochem. 8:256–264, 1997) © *Elsevier Science Inc. 1997*

**Keywords:** zinc availability; dose-rate idiorrhythm; calcified tissue; slope-ratio assay; weanling rat

## Introduction

Human and animal dietary intakes of zinc under free living conditions are not constant but vary depending upon dietary habits, food availability, and affordability.[1] Although the space-time variability in food and nutrient consumption is self-evident, the relevance of such a dose-rate variability in dietary zinc intake for zinc nutritional status, as well as for the other trace elements, remains unknown or ignored.[2] Zinc absorption is now considered to be a dose-dependent, carrier-mediated, saturable process where uptake at the brush border membrane is the rate-limiting step so that a relatively higher percentage of zinc is absorbed from the diet when the amount of available zinc is decreased as compared to when available zinc is increased.[3] Thus, zinc absorption readily responds to variability in dietary zinc so that an intake of only one third of the recommended dietary allowances does not cause serum or urinary zinc concentrations to fall in young men[4] nor induces a negative balance in lactating women.[5] Although a prolonged marginal zinc deficiency does not impair the functional role of endogenous zinc excretion in zinc homeostasis, the efficiency of

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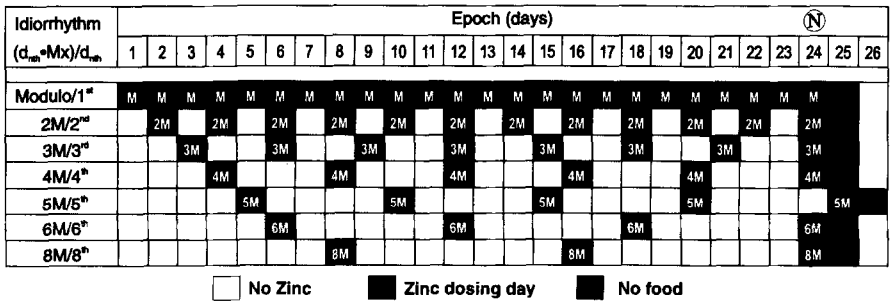


**Figure 1** Idiorrhythmic feeding experimental design. Idiorrhythm ($I$)(dose-rate); $I = [d_{nth}(Mx)]/d_{nth}$ = mg $Zn\ kg^{-1}\ d_{nth}^{-1}$, $d_{nth}$ = sequential number of zinc dosing day. Epoch ($E$) = 24 day experimental period. Modulo ($Mx$)(dose-time equivalent) = mg of $Zn \cdot kg^{-1} \cdot d^{-1}$ or mg of $Zn \cdot kg^{-1} \cdot E^{-1}$]; $M3$ = 3 mg of $Zn \cdot kg^{-1} \cdot d^{-1}$ or 72 mg of $Zn \cdot kg^{-1} \cdot E^{-1}$, $I$ = 3/1, 6/2, 9/3, 12/4, 15/5, 18/6, and 24/8; $M6$ = 6 mg of $Zn \cdot kg^{-1} \cdot d^{-1}$ or 144 mg of $Zn \cdot kg^{-1} \cdot E^{-1}$, $I$ = 6/1, 12/2, 18/3, 24/4, 30/5, 36/6, and 48/8; $M12$ = 12 mg of $Zn \cdot kg^{-1} \cdot d^{-1}$ or 288 mg of $Zn \cdot kg^{-1} \cdot E^{-1}$, $I$ = 12/1, 24/2, 36/3, 48/4, 60/5, 72/6, and 96/8; M24 = 24 mg of $Zn \cdot kg^{-1} \cdot d^{-1}$ or 576 mg of $Zn \cdot kg^{-1} \cdot E^{-1}$, $I$ = 24/1, 48/2, 72/3, 96/4, 120/5, 144/6, and 192/8.

zinc absorption cannot be sustained and decreases when a zinc-restricted diet is continued for as long as 6 months.[6] These data show that the variability in dietary zinc consumption results in different metabolic pool sizes and therefore, as a result of homeostasis, the quantity of zinc necessary to maintain the existing pool of body zinc will vary.[7]

Because the logic of cycles and other temporal effects are not the same as the logic of linear and monotonic trends,[8] an idiorrhythmic feeding regimen was recently proposed to study the impact of dose-rate in trace element nutrition.[9,10] An idiorrhythmic feeding regimen refers to a distinctly proportional and regularly recurring pattern of nutrient intake in which the nutrient dose is coupled to frequency so that their product, i.e., dose-time equivalent modulo, is kept constant over a selected period of time. Whereas, the actual dose and frequency, i.e., dose-rate idiorrhythm, varies regularly according to a predetermined pattern. Viewing the dose over the time span of the entire experimental epoch as a dose-time equivalent modulo, allows the dose to be divided into a series of equal products or idiorrhythms of different dose size with different frequency. Then, they are administered in a regularly recurring pattern to allow the same total dose as if the animals were fed zinc every day, albeit at a different rate. In the simplest form of idiorrhythmic feeding, the periods of feeding the nutrient are separated by periods without the nutrient, or by a different amount of the nutrient. Idiorrhythmic feeding is a precisely defined model for dose-rate studies in nutrition and is in clear distinction to other predominantly descriptive concepts related to meal and nutrient partitioning. It was found that a zinc dose-rate idiorrhythm induced changes in metabolic efficiency, which in turn generated a complex, gestalt-like, biphasic pattern of growth response where an intake of dietary zinc exceeding requirements had a limited capacity to compensate for a previously deficient zinc intake.[10] That capacity was dependent both on the dose-time equivalent modulo and on the dose-rate idiorrhythm. In other experiments (accepted in *Br. J. Nutr.*), we showed that idiorrhythmic feeding changed zinc deposition in the femur and incisor without affecting metallothionein induction in the intestine, or restraint-induced expression of heat shock protein 70 mRNA in the aorta of weanling rats. The metabolic efficiency of bone zinc deposition was dependent on the zinc dose-time equivalent modulo and zinc dose-rate idiorrhythm in a non-linear fashion, which indicated that the difference in metabolic efficiency was caused by partitioning of metabolizable zinc.

The aim of the present experiment was to quantitatively assess changes in the metabolic availability of zinc for its deposition in bone and teeth of animals subjected to idiorrhythmic dose-rate variability in dietary zinc by using a slope-ratio assay of the analogous idiorrhythms, i.e., the idiorrhythms of different dose size, but the same dosing frequency.[10] It has been demonstrated already that femur zinc is the best indicator for assessing the biological availability of dietary zinc[11] and that the latter can be quantitatively assessed by the slope-ratio assay.[12]

## Methods and materials

### *Idiorrhythmic dose-rate experimental design*

Idiorrhythm ($I$) describes a distinctly proportional and regularly recurrent pattern. In this case, it refers to the feeding of a specific nutrient, zinc. Idiorrhythm requires that the total dose-time equivalent, called modulo ($Mx$), is kept constant over the entire time of the experiment or epoch ($E$); the size of the nutrient dose (peak dose concentration) and nutrient dose frequency (time interval between the doses), i.e., the dose-rate, varies regularly according to a predetermined pattern, or idiorrhythm. Thus, for any given modulo, the size and the frequency of the dose are varied such that the total dose is constant, i.e., the product of the dose size and the dose frequency is constant. In such an idiorrhythmic model, periods of feeding diets containing the nutrient are separated by periods of feeding a diet deficient in the nutrient (*Figure 1*). Continuous or daily feeding of a nutrient may be regarded as a special case of idiorrhythm where the time base is only one day. Thus, an idiorrhythm is not the diet, but it is inseparable from the diet because of the coupling of dose to frequency.[10] The relationship between dose-rate idiorrhythm, selected dose-time equivalent modulo level ($Mx$; $x$ = mg $Zn \cdot kg^{-1}$ diet $\cdot d^{-1}$), and sequential number of the day on which the peak dose is administered, i.e., dosing day ($d_{nth}$), is expressed as: $I = [d_{nth}(Mx)]/d_{nth}$ = constant.

Idiorrhythmic coupling of dose to frequency in a regularly recurrent pattern insures that the animals fed by any one of the idiorrhythms belonging to the same dose-time equivalent modulo, will receive the same amount of nutrient over the entire idiorrhythmic epoch, albeit at a different dose-rate. The amount of dietary zinc on a zinc dosing day is regarded as the dose-rate amplitude, whereas, the number of zinc dosing days over the epoch is regarded as the dose-rate frequency. All the idiorrhythms sharing the same zinc dosing day ($d_{nth}$) are considered analogous regard-

**Table 1** Basal zinc-deficient diet*

| Ingredients | g/kg |
|---|---|
| Cornstarch | 427.495 |
| Egg white (80% Protein) | 200.00 |
| Dextrinized starch (90% Tetrasaccharides) | 100.00 |
| Dextrose | 100.00 |
| Soybean oil | 75.00 |
| Fiber (Solka Floc) | 50.00 |
| Mineral mix (excluding Zn)† | 35.00 |
| Vitamin mix‡ | 10.00 |
| Choline bitartrate | 2.50 |
| Tert-butylhydroquinone (TBHQ) | 0.005 |

*Contains <0.6 mg Zn/kg as assessed by inductively coupled argon plasma atomic emission spectrometry (Ref. 15).
†*Mineral Mix* (g/Kg): $CaCO_3$ (anhydrous), 203.76; $CaHPO_4$, 332.27; $KH_2PO_4$, 126.79; MgO, 23.68; $Na_2SiO_3 \cdot 9H_2O$, 7.25; $FeSO_4 \cdot 7H_2O$, 4.98; $MnCO_3$, 0.63; $CuCO_3 \cdot Cu(OH)_2$, 0.30; $KCr(SO_4)_2 \cdot 12H_2O$, 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; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0106; $Na_2SeO_4$ (anhydrous), 0.01025; $KIO_3$, 0.010; dextrose to 1000.
‡*Vitamin Mix* (AIN-93 modified) (g/Kg): Nicotinic acid, 3.00; Ca pantothenate, 1.00; pyridoxine · HCl, 0.70; thiamin · HCl, 0.60; riboflavin, 0.60; folic acid, 0.20; D-biotin, 0.02; vitamin B-12 (0.1% in mannitol), 2.50; DL-*a*-tocopheryl acetate, 15.00; retinyl palmitate, 0.80; cholecalciferol, 0.25; phylloquinone, 75.0 mg; dextrose to 1000 g.

less of their zinc dose-time equivalent modulo level *(Mx)*. To facilitate the comparison of such analogous idiorrhythms, Mx was added as a subscript for I ($I_{Mx}$).

## Diets

The composition of the basal diet without zinc is shown in *Table 1*.[13] The diet was based on eggwhite protein supplemented with biotin. Zinc carbonate and all other mineral supplements were reagent grade (J.T. Baker, Phillipsburg, NY, and Pfaltz and Bauer, Inc., Waterbury, CT USA).* The basal diet closely resembled the AIN-93G diet for growing rats[14] except that the mineral mix was reformulated to meet the requirements for phosphorus when eggwhite is used as the source of protein. The diet contained less than 0.6 mg Zn/kg as assessed by inductively coupled argon plasma atomic emission spectrometry (ICAP-AES).[15] 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.

Four representative dose-time equivalent zinc modulos were chosen over a 24 d idiorrhythmic epoch. The four Mx were, zinc-deficient (M3; 3 mg of $Zn \cdot kg^{-1} \cdot d^{-1}$), moderately zinc-deficient (M6; 6 mg of $Zn \cdot kg^{-1} \cdot d^{-1}$), adequate (M12; 12 mg of $Zn \cdot kg^{-1} \cdot d^{-1}$), and ample (M24; 24 mg of $Zn \cdot kg^{-1} \cdot d^{-1}$). Each Mx had seven analogous dose-rate idiorrhythms arranged in an increasing order: (a) I = *Mx/1; 3, 6, 12,* or 24 mg of $Zn \cdot kg^{-1} \cdot d_1^{-1}$ fed daily, (b) *I = 2Mx/2; 6, 12, 24,* or 48 mg of $Zn \cdot kg^{-1} \cdot d_2^{-1}$ fed every other day and separated by a day of feeding a diet with no zinc, (c) *I = 3Mx/3; 9, 18, 36,* or 72 mg of $Zn \cdot kg^{-1} \cdot d_3^{-1}$ fed every third day and separated by two days of feeding a no-zinc diet, (d) *I = 4Mx/4; 12, 24, 48,* or 96 mg of $Zn \cdot kg^{-1} \cdot d_4^{-1}$ fed every fourth day and separated by three days of feeding a no-zinc diet, (e) I = *5Mx/5; 15, 30, 60,* or 120 of mg $Zn \cdot kg^{-1} \cdot d_5^{-1}$ fed every fifth day and separated by four days of feeding a no-zinc diet, (f) I = 6Mx/6; 18, 36, 72, or 144 mg of $Zn \cdot kg^{-1} \cdot d_6^{-1}$ fed every sixth day and separated by five days of feeding a no-zinc diet, and (g) I = 8Mx/8; 24, 48, 96, or 192 mg of $Zn \cdot kg^{-1} \cdot d_8^{-1}$ fed every eighth day and separated by seven days of feeding a no-zinc diet. Each zinc dose-time equivalent Mx was comprised of seven analogous zinc dose-rate idiorrhythms and was run as an independent experiment. The expected vs. analyzed

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**Table 2** Zinc concentration of experimental diets fed idiorrhythmically,— expected (Ex) versus analyzed (An) values of zinc supplemented to basal zinc-deficient diet as zinc carbonate*

| | Modulo‡ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | M3 | | M6 | | M12 | | M24 | |
| | (mg Zn · kg$^{-1}$ · d$^{-1}$) | | | | | | | |
| Idiorrhythm† (mg Zn · kg$^{-1}$ · $d_{nth}^{-1}$) | Zinc Deficient | | Moderate Zinc Deficient | | Zinc Adequate | | Zinc Ample | |
| | Ex | An | Ex | An | Ex | An | Ex | An |
| | (mg Zn/kg of diet) | | | | | | | |
| 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 |

*Values are means ± SD, n = 3.
†Idiorrhythm (I)(dose/rate); I = ($d_{nth}$Mx)/$d_{nth}$ = mg of Zn · kg$^{-1}$ · $d_{nth}^{-1}$; $d_{nth}$ = sequential number of the zinc dosing day.
‡Modulo (Mx) = dose/time equivalent [mg of Zn · kg$^{-1}$ · d$^{-1}$ or mg of Zn · kg$^{-1}$ · epoch (E)$^{-1}$]; M3 = 3 mg Zn · kg$^{-1}$ · d$^{-1}$ or 72 mg of Zn · kg$^{-1}$ · E$^{-1}$, I = 3/1, 6/2, 9/3, 12/4, 15/5, 18/6, and 24/8; M6 = 6 mg of Zn · kg$^{-1}$ · d$^{-1}$ or 144 mg of Zn · kg$^{-1}$ · E$^{-1}$, I = 6/1, 12/2, 18/3, 24/4, 30/5, 36/6, and 48/8; M12 = 12 mg of Zn · kg$^{-1}$ · d$^{-1}$ or 288 mg of Zn · kg$^{-1}$ · E$^{-1}$, I = 12/1, 24/2, 36/3, 48/4, 60/5, 72/6, and 96/8; M24 = 24 mg of Zn · kg$^{-1}$ · d$^{-1}$ or 576 mg of Zn · kg$^{-1}$ · E$^{-1}$, I = 24/1, 48/2, 72/3, 96/4, 120/5, 144/6, and 192/8.

zinc contents of all 28 idiorrhythmic experimental diets are shown in *Table 2*.

### *Animals*

The study was approved by the Animal Use Committee of the USDA, ARS, Grand Forks Human Nutrition Research Center and was in accordance with the guidelines of the National Research Council[16] on the experimental use of laboratory animals.

Twenty-one-day-old, male Sprague-Dawley rats (Sasco, Omaha, NE USA) were housed in individual stainless steel cages with a mesh floor and located in a room controlled for temperature- (22 to 24°C) and humidity- (44–55%), and on 12 hr light/12 hr dark cycle. They were given free access to a powdered diet and deionized water (Super Q System, Millipore Corp., Bedford, MA USA). After having been fed their respective diets for 24 days and deprived of food overnight (16 hr), the animals were killed by halothane inhalation. The dry ash of the left femur and a single upper incisor were analyzed for zinc content[11,12] by ICAP-AES.[15]

### *Statistical analysis*

Results are expressed as means ± SD for 8 rats/group. The effects of four different dietary zinc dose-time equivalent *Mx*, each one with seven analogous zinc dose-rate idiorrhythms on mineral content of the femur and the incisor were assessed by ANOVA. The Ryan-Einot-Gabriel-Welsh (REGW) multiple $F$ test[17] was used to determined if the mean values between the idiorrhythms of the same *Mx* were significantly different. The metabolic availability of zinc for deposition in the femur and incisor was quantitatively assessed by a slope-ratio assay of the M3, M6, and M12 analogous idiorrhythms. Šidák contrasts[18] were used for pairwise comparisons of the slope of the analogous zinc dose-rate idiorrhythms. Differences between groups for both tests were considered significant if $P \leq 0.05$.

## Results

The results of the effect of the idiorrhythmic dose-rate variability in dietary zinc on the deposition of zinc in the femur are shown in *Figure 2*. This figure also demonstrates how the results of the idiorrhythmic dose-rate feeding, as shown in the center of the three-dimensional board, can be analyzed with a set of projection panels along its sides and with respect to the dose-rate idiorrhythm or time ($x$-axis), dose-time equivalent modulo or dose size ($y$-axis), and the corresponding response of the indicator tissue, i.e., femur ($z$-axis). The numbering of doses along the $y$-axis corresponds to the expected dietary zinc values as shown in *Figure 1*, whereas the values on the board in *Figure 2* are the actual analyzed dietary zinc values.

The dose related effects of idiorrhythmic dose-rate feeding on zinc deposition in the femur for the dose-time equivalent M3, M6, M12, and M24 are shown in the projection panels on the left hand side ($y$-axis). The amount of zinc deposited in the femur increased with an increase in the size of the dose-time modulo equivalent, M3 < M6 < M12 < M24. However, except for M3, this increase was not linear across the idiorrhythms, but biphasic. This occurred in spite of the fact that the product of dose with frequency was constant for each of the idiorrhythms within the same dose-time equivalent modulo.

The time related effects of idiorrhythmic dose-rate feeding on zinc deposition in the femur for the same dose-time equivalent M3, M6, M12, and M24 are shown in the projection panels ($x$-axis) on the right hand side of *Figure 2*. These data showed that the biphasic response was indeed time-related. In contrast to the M3 idiorrhythms, M6, M12, and M24 idiorrhythms had maximal depression points in femur zinc deposition when the animals were fed by the $I = 4Mx/4$, i.e., either 24, 48, or 96 mg of dietary $Zn \cdot kg^{-1} \cdot d_4^{-1}$ fed every fourth day separated by three days without zinc. Thereafter, the amount of femur zinc increased for $I = 5Mx/5$ and reached a secondary peak for $I = 6Mx/6$, where the values approached those of rats receiving continuous daily doses of dietary zinc. Then, zinc deposition in the femur dropped again for $I = 8Mx/8$, the latter values overlapped those of $I = 4Mx/4$.

Thus far, both the dose and the time component analysis of the effects of the idiorrhythmic dose-rate variability in dietary zinc were done within the frame of constant dose-time frequency. That is, neither dose nor frequency were invariant with the sequential progression of the idiorrhythmic series. The set of analogous idiorrhythm projection panels in the right rear of *Figure 2* shows that the effect of idiorrhythmic feeding on femur zinc deposition can be analyzed as a series of sets of the analogous idiorrhythms, i.e., the idiorrhythms of the same time base but belonging to different *Mx*.

The results clearly demonstrate the importance of the time component in the idiorrhythmic zinc dose-rate variability of dietary zinc. When animals were fed zinc continuously ($I = Mx/1$), zinc deposition in the femur was clearly dose dependent. However, when zinc was fed at a rate four times the continuous daily amount, but every fourth day ($I = 4Mx/4$), the amount of zinc in the femur did not differ whether the animals were fed 12, 24, 48, or 96 mg of $Zn \cdot kg^{-1}$ diet $\cdot d_4^{-1}$. The amount of zinc in the femur "fanned out" again for $I = 5Mx/5$ and $I = 6Mx/6$ before they come together again for $I = 8Mx/8$.

Essentially, the analogous idiorrhythm allows for what can be tentatively named "the dosing of time" where the time component on the abscissa of a given idiorrhythm is an independent variable and the ordinate of the femur dose-response across the different *Mx* is a dependent variable. Thus, each set of analogous idiorrhythms can be analyzed as an independent dose-response set which then can be quantitatively compared with yet another set of the analogous idiorrhythms by the standard slope-ratio assay. In more familiar terms, such an approach towards the quantitative assessment of the effect of variability in dietary zinc allows us to treat each set of analogous idiorrhythms as if they were a different source of zinc whose metabolic availability we would like to compare.

And that is exactly what we did. In *Figure 3* we compared the dependence of femur and incisor zinc deposition across M3, M6, and M12 on any given idiorrhythm from $I = Mx/1$ to $I = 8Mx/8$. These results showed that the metabolic availability of zinc for deposition in the femur and incisor depended on the time component of the idiorrhythm, and had a similar pattern for both calcified tissues. The data for the M24 idiorrhythms were not included in the analysis because the visual examination of plots showed them to deflect from those for the M3-M6-M12 directional vector. Indeed, M24 data "fanned" out from left to right

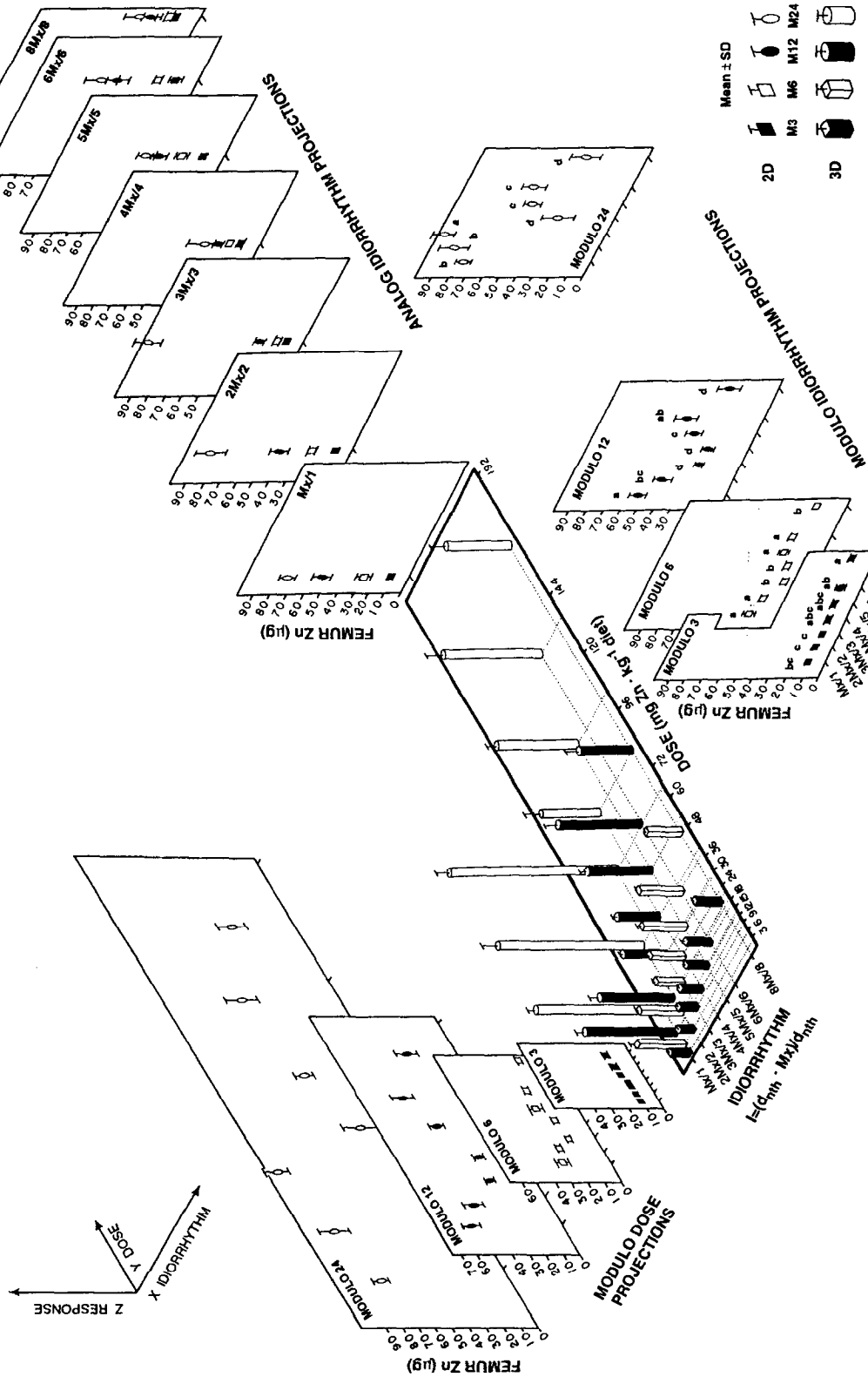


**Figure 2** Three-dimensional (3D) projection analysis of the impact of zinc dose-rate idiorrhythm (I) and zinc dose-time equivalent modulo (M) on zinc deposition in the femur of weanling male rats. The central board shows *I* on the *x*-axis, M on the *y*-axis, and femur zinc response on the *z*-axis. The sides of the 3D board shows M*x* projection panels on the left, *I* projection panels to the right, and analogous *I* projection panels to the right rear. Values are means ± SD, $n = 8$. See legend of *Figure 1* for descriptions of I, E and M.

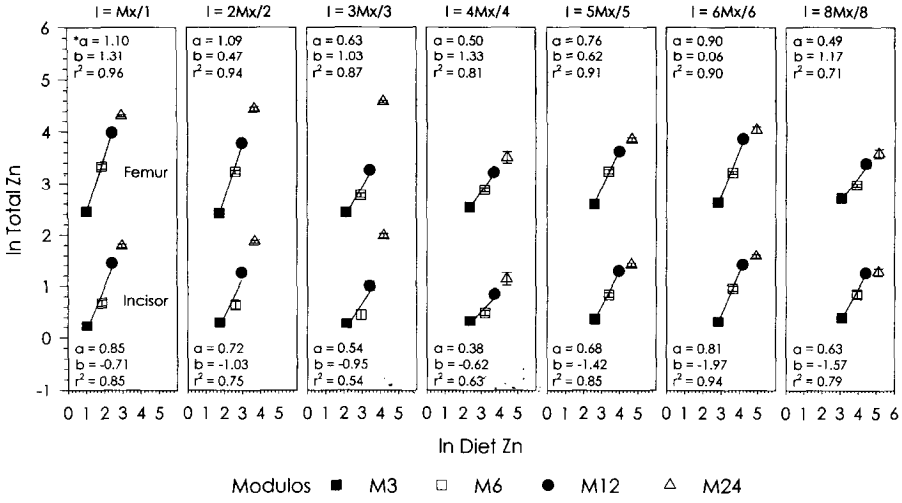


**Figure 3** Slope-ratio assay analysis of the effect of analogous zinc dose-rate idiorrhythms upon metabolic availability of zinc for deposition in the femur (upper regression line) and incisors (lower regression line) of male weanling rats. Each block represents the set of M3, M6, and M12 analogous idiorrhythms: *I* = *Mx*/1, *I* = 3/1, 6/1, and 12/1; *I* = *2Mx*/2, *I* = 6/2, 12/2, and 24/2; *I* = *3Mx*/3, *I* = 9/3, 18/3, and 36/3; I = *4Mx*/4, I = 12/4, 24/4, 48/4; *I* = *5Mx*/5, *I* = 15/5, 30/5, and 60/5; *I* = *6Mx*/6, *I* = 18/6, 36/6, and 72/6; and *I* = *8Mx*/8, *I* = 24/8, 48/8, 96/8. Note that M24 analogous idiorrhythm data were not included in regression analysis. Values are means ± SD, *n* = 8. *a and b represent the constants of the general equation; ln *y* = *a*ln *x* + *b*. See legend of *Figure 1* for descriptions of I, E, and M.

with the progression of the idiorrhythmic series from *I* = *2Mx/2* to *I* = *8Mx/8*. Recently we also showed that the deposition of zinc in the femur and incisor of continuously fed animals can be described by a broken-line assay,[19] where zinc deposition has its fast upward component at concentrations of up to about 15 mg Zn/kg. Thereafter, a very slow linear increase almost parallel to the *x*-axis was observed.† Therefore, the M24 data set would belong to the slow arm of the broken-line assay and, indeed, they were skewed to the right as already predicted from the broken-line assay model. However, the changes seen in either the slope response pattern or correlation coefficients, when all four modulos were included in the analysis, did not appreciably differ from those for the three modulo analysis presented in *Figure 3* (data not shown).

The slopes progressively decreased with an increase in the idiorrhythm until *I* = *4Mx/4*, where the metabolic availability was about one half that of animals fed zinc daily (*I* = *Mx/1*). Thereafter, the metabolic availability of zinc for femur and incisor deposition increased, and for *I* = *6Mx/6*, it again reached nearly that of animals fed zinc daily. The final drop in zinc deposition for *I* = *8Mx/8* was considerably larger in femurs than in incisors. The statistical differences between the pairs of slopes of analogous idiorrhythms are shown in *Table 3*. The results clearly show that the metabolic availability of zinc for deposition in the femur and incisor depends considerably on the variability in dietary zinc supply from the same food source.

It should be mentioned that the data for the idiorrhythmic experiment can be further analyzed as a set of analogous doses as it was for the analogous idiorrhythms. However, we had only one set of data with the same dose but different idiorrhythms, i.e., $I_{M3}$ = 24/8, $I_{M6}$ = 24/4, $I_{M12}$ = 24/2, and $I_{M24}$ = 24/1. This dilutional type of diet series seems to be slightly different from the classical dose-response or dose-analogous idiorrhythmic series $I_{M3}$ = 3/1, $I_{M6}$ = 6/1, $I_{M12}$ = 12/1, and $I_{M24}$ = 24/1.

†Momčilović, B., Blake, M.J., and Reeves, P.G. (1995) Aortal heat shock protein-70mRNA and intestinal metallothionein response in weanling rats fed graded levels of dietary zinc. *FASEB J.* 9: A738 (abst).

## Discussion

The results of this experiment showed that: (a) zinc deposition in the femur and incisor of weanling rats depends on zinc dose-rate idiorrhythm variability in dietary zinc and zinc dose-time equivalent modulo in a non-linear fashion, (b) the impact of the idiorrhythmic feeding scheme can be quantified by a slope-ratio assay of the analogous idiorrhythms, i.e., the idiorrhythms of the same time base but belonging to different modulos, and (c) the time component of zinc dose-rate idiorrhythm changes the metabolic availability of zinc for deposition in the femur and incisor by as much as 50% when zinc is dosed every fourth day.

Our results demonstrated that both dose and time had an effect on the response. The classical dose-response model ignores the time component and therefore cannot resolve the dose effect from the time effect. This oversimplifies the classical model and renders it suitable only for accurate description of a limited number of special and/or extreme cases. On the other hand, by coupling dose to frequency, the

**Table 3** Šidák contrast-*P* values for data shown in *Figure 3*. Pairwise comparison of the slope of analogous zinc dose-rate idiorrhythms for zinc dose-time equivalent M3, M6, and M12

| Analogous Idiorrhythms | Analogous Idiorrhythms | | | | | |
|---|---|---|---|---|---|---|
| | 2Mx/2 | 3Mx/3 | 4Mx/4 | 5Mx/5 | 6Mx/6 | 8Mx/8 |
| Femur | | | | | | |
| Mx/1 | NS* | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 |
| 2Mx/2 | | <0.05 | <0.05 | <0.05 | <0.10 | <0.05 |
| 3Mx/3 | | | NS | NS | <0.05 | NS |
| 4Mx/4 | | | | <0.05 | <0.05 | NS |
| 5Mx/5 | | | | | NS | <0.05 |
| 6Mx/6 | | | | | | <0.05 |
| Incisor | | | | | | |
| Mx/1 | NS | <0.10 | <0.05 | NS | NS | NS |
| 2Mx/2 | | NS | <0.05 | NS | NS | NS |
| 3Mx/3 | | | NS | NS | <0.05 | NS |
| 4Mx/4 | | | | <0.05 | <0.05 | <0.05 |
| 5Mx/5 | | | | | NS | NS |
| 6Mx/6 | | | | | | NS |

*NS not significant; *P* < *0.05* significant, *P* < *0.10* marginally significant.

idiorrhythmic dose-rate model brings the dimension of time to the dose-response and can therefore be classified as a time-sensitive experimental design.[8] The idiorrhythm is like the Janus with two faces, i.e., dose and frequency coupled in endless multiples of possible relationships. The idiorrhythmic experimental design allows us to pick up, in particular, any such relationship of duality out of the stream of change and to "freeze" it as an idiorrhythm for subsequent analysis. Such a model can be used for active exploration of the time dynamics of any real system and, in contrast to computer simulation models, does not require very detailed preconceptions of unknown dynamics of the system under consideration.[8]

Our results showed that externally imposed time-dependent oscillations in the nutrient dose with frequency have a definitive impact on the operation of intermediary metabolism. We think that such an effect of dose-rate in a biological system is mediated through a number of simultaneous steady-states exhibiting relaxation oscillation via rapidly changing states. Heinrich et al.[20] defined relaxation oscillation as a periodic alternation between slow and rapid motion in a quasi steady state. Biological systems are hierarchical with respect to time and material structure. The time hierarchy is necessary because systems simultaneously involve many reactions that are dependent on each other and take place with different velocities.[20] The biological hierarchy, with respect to time, ranges over more than 15 orders of magnitude,[21] but in modeling of metabolism, it is usually restricted to a range of about five to six orders of magnitude from $10^{-2}$ seconds to $10^{4}$ seconds,[20] the latter being less than three hours. However, the time sensitive idiorrhythmic dose-rate feeding regimen can operate on a time scale of days or weeks. This makes it suitable for the study of time related nutrient effects at the level of the whole organism.

It is generally assumed that gastrointestinal zinc absorption is essentially completed by four hours after ingestion.[22] In the rat, the gut adapts to the diet primarily by changing its epithelial mass over time,[23] and any impairment of absorption caused by luminal deprivation of nutrients is rapidly corrected within 15 to 24 hr of refeeding.[24] However, in idiorrhythmic feeding, the animals are not deprived of food between dosing days, but receive the zinc-deficient diet instead. The appearance of a secondary peak in metabolic zinc utilization excludes the possibility that the change in intestinal cell mass plays a role in the availability of the idiorrhythmically administered zinc. The absorption of $^{65}Zn$ by zinc-deficient rats was shown to continue at a maximal rate for 96 hr. On the other hand, in animals with adequate dietary zinc, improved absorption lasted for only 20 hr. This caused the magnitude of zinc absorption to be greater in the deficient rats than in controls.[25] In the present experiment, the metabolic efficiency of idiorrhythmically administered zinc dropped progressively in rats fed by $I = Mx/1$ to $I = 4Mx/4$, where it reached its lowest value precisely after 96 hr of zinc deprivation. Thus, at the point where Schwarz and Kirchgessner[25] proposed that zinc absorption was operating at its greatest rate, idiorrhythmic feeding showed that the metabolic deposition of zinc into calcified tissue was at its lowest rate. This indicates that either enteral turnover of zinc, but not true absorption, was increased, or that the metabolic partitioning between different tissues of the body was affected at the expense of the calcified tissues, or both.

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, could result only from a change in the zinc dose-rate idiorrhythm. In other words, a change in zinc deposition in the indicator tissues, femur, and incisor, is the common vector of all changes that may occur in different compartments of the whole animal.

A cysteine-rich protein (CRIP) was recently identified as a peptide that occurs primarily in the intestine and binds zinc in the mucosa during absorption.[26] High dietary zinc does not affect CRIP concentration, but it greatly increases metallothionein (MT) that may compete with CRIP to decrease zinc absorption.[27] However, we have found (data to be reported in *Br. J. Nutr.*) that an idiorrhythmically administered dose of zinc identical to that in this experiment did not induce an increase in intestinal MT. This suggests that the idiorrhythmic change in zinc deposition in calcified tissue of intact animals is not a secondary consequence of binding of zinc by enhanced intestinal MT.

The dynamics of small intestinal cells is well known. They arise in the crypt between villi, migrate up towards the tip of the villus and are shed into the lumen after about three to six days in the human and 2 to 3 days in the rat.[28] The dynamics of epithelial turnover in the small intestine is considered to be regulated by apoptosis. Therefore, the cells are programmed to die and are not summarily pushed to their death by the younger generations coming from below.[29] The secondary peak of zinc deposition in the femur and incisor appeared after 5 to 6 days, after about two life cycles of the mucosal epithelial cells.[28] Therefore, time dependent changes in metabolic efficiency of idiorrhythmic zinc dose-rate feeding cannot be attributed to cell dynamics in the gastrointestinal villi. Zinc, like lead, cadmium, mercury, and manganese,[30] does not seem to be merely trapped in the intestinal mucosa waiting to be desquamated into the intestinal lumen.

Because the efficiency of zinc absorption is inversely related to the amount of zinc in the diet, Sandstrom[3] speculated that the distribution of zinc intake across daily meals can influence zinc utilization. In a human study, Jackson et al.[31] found that when dietary zinc was changed, there was a delay before balance was re-established. They attempted to relate this to the rate of exchange between the half-life of labeled plasma zinc and tissue zinc pools rather than to the excretion of zinc from the body, an idea widely adopted by that time.[7] However, the same authors[31] were not able to explain what mechanism could account for a decrease in the proportion of absorbed zinc when a second dose of zinc of the same size was administered to the same subject 4 days after the first. This parallels our results showing that the $I = 4Mx/4$ pattern of feeding decreased zinc metabolic efficiency. This indicates that the same time-dependent mechanism that accounts for the 50% reduction in the metabolic efficiency of zinc relative to daily feeding in the rat, may operate as well in the human. This may occur regardless of the difference in the mucosal turnover rate between the two species.

In this report we showed that the slope-ratio assay can be

used for the quantitative assessment of changes in zinc metabolic activity induced by the zinc dose-rate idiorrhythm over a range of zinc intakes. Hurley and Swenerton[32] showed a lack of bone and liver zinc mobilization under teratogenic conditions of zinc deficiency. Later, Murray and Messer[33] showed that the skeleton acts as an avid scavenger of circulating zinc, rather than a reservoir that can be alternatively filled or depleted. Therefore, all the evidence supports the conclusion that calcified tissue effectively mirrors the temporal metabolic variability in the availability of dietary zinc induced by idiorrhythmic dose-rate feeding. As shown in this experiment, the occurrence of a secondary peak of zinc deposition in the femur and incisor when zinc was dosed every fifth or sixth day, and that of secondary body growth peak demonstrated in the previous experiment,[10] may be interpreted as an interaction between the extrinsic idiorrhythm of nutrient intake and an underlying intrinsic infradian rhythm (longer than 1 day) in the body. A large number of infradian rhythms have been observed in weanling rats, one of them with a duration of 5.4 days.[34] This appears to synchronize with our $I = 5Mx/5$ and $I = 6Mx/6$ zinc dosing schedules.

We think it is important to emphasize that under the conditions of 24-day idiorrhythmic feeding, and for the four different modulo dose-time equivalents tested, none of the dosing schedules was superior to that of daily intake of zinc. Indeed, the total amount of zinc in the diet was the key determinant of how much zinc reached the bone, whereas the time between the doses can dramatically impair the metabolic efficiency of the administered zinc. Apparently, any assessment of the success of a non-fuel nutrient supplementation program for humans should include: (a) careful scrutiny of the amount of nutrient that may be ingested between nutrient dosing days in order to control for the total intake of the nutrient, and (b) knowledge of the timing of underlying metabolic processes that may critically affect the efficiency of nutrient utilization. If the amount of nutrient between supplementation days is not taken into account, then the effects of intermittent or sporadic supplementation may appear to be more effective than if the nutrient were fed on a daily basis. This observation may help explain the recent contradictory results in experiments that used intermittent iron supplementation. Viteri et al.[35] showed that true absorption and retention of supplemental iron was more effective when iron was administered to normal and iron-deficient rats every 3 days than when administered daily. On the other hand, Cook and Reddy[36] found no significant advantage in giving iron less often than once a day to humans. Our results show that no meaningful interpretations concerning bioavailability and intermittent supplementation are possible unless the spatio-temporal variability in nutrient intake, i.e. dose-rate, is considered.

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## APPENDIX I

Comparative analysis of slope-ratio assay with or without M24

| | Femur | | Incisor | |
|---|---|---|---|---|
| Idiorrhythm | −M24 | +M24 | −M24 | +M24 |
| General equation: $\ln y = a \ln x + b$ | | | | |
| Mx/1 | $\ln y = 1.10 \ln x + 1.31$ | $0.99 \ln x + 1.48$ | $0.84 \ln x - 0.71$ | $0.84 \ln x - 0.70$ |
| 2Mx/2 | $1.08 \ln x + 0.49$ | $1.07 \ln x + 0.53$ | $0.72 \ln x - 1.03$ | $0.84 \ln x - 1.30$ |
| 3Mx/3 | $0.63 \ln x + 1.04$ | $1.06 \ln x - 0.09$ | $0.54 \ln x - 0.95$ | $0.86 \ln x - 1.79$ |
| 4Mx/4 | $0.51 \ln x + 1.32$ | $0.49 \ln x + 1.37$ | $0.37 \ln x - 0.60$ | $0.42 \ln x - 0.73$ |
| 5Mx/5 | $0.77 \ln x + 0.61$ | $0.62 \ln x + 1.06$ | $0.68 \ln x - 1.42$ | $0.54 \ln x - 0.99$ |
| 6Mx/6 | $0.90 \ln x + 0.05$ | $0.71 \ln x + 0.68$ | $0.81 \ln x - 1.98$ | $0.63 \ln x - 1.37$ |
| 8Mx/8 | $0.49 \ln x + 1.18$ | $0.45 \ln x + 1.31$ | $0.63 \ln x - 1.57$ | $0.45 \ln x - 0.92$ |

Comparative analysis of $r^2$ with or without M24

| | Femur | | Incisor | |
|---|---|---|---|---|
| Idiorrhythm | −M24 | +M24 | −M24 | +M24 |
| Mx/1 | 0.96 | 0.97 | 0.85 | 0.91 |
| 2Mx/2 | 0.94 | 0.97 | 0.75 | 0.89 |
| 3Mx/3 | 0.87 | 0.89 | 0.54 | 0.82 |
| 4Mx/4 | 0.81 | 0.82 | 0.63 | 0.68 |
| 5Mx/5 | 0.91 | 0.90 | 0.85 | 0.85 |
| 6Mx/6 | 0.90 | 0.88 | 0.94 | 0.90 |
| 8Mx/8 | 0.71 | 0.76 | 0.79 | 0.71 |

It should be noted that the correlation statistics were not improved at all by inclusion of M24 data although the sample size was increased by 25% and the length of the slope vector doubled. The finding indicates the boot-strapping of the slope with M24 data.