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Idiorrhythmic zinc dose-rate induction of intestinal metallothionein in rats depends upon their nutritional zinc status†

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Abstract

The idiorrhythmic dose-rate feeding experimental model was used to study the induction of intestinal metallothionein (iMT) by zinc (Zn) in the gastrointestinal (GIT) mucosa of young growing male rats relative to their nutritional Zn status. The idiorrhythmic approach requires that the average dietary Zn concentration, referred to as modulo (M), is kept constant across different groups over the whole experimental epoch (E). This is done by adjusting the Zn concentration of the supplemented diet to compensate for the reduction in the number of days on which this diet is fed, the latter being spread evenly over the whole experiment. Idiorrhythms (I) involve offering the diet with *n* times the overall Zn concentration (M) only every *n*th day with a Zn-deficient diet offered on other days. We studied three modulos (low-Zn, M3; adequate-Zn, M12; and high-Zn, M48), each M having 8 analogous idiorrhythms (I = Mx/1 to 8Mx/8); every I was fed over a 48-d idiorrhythmic E. Over the wide range of peak doses of dietary Zn (3–384 mg Zn/kg diet), the higher the modulo, the greater the capacity for iMT to be induced (M3 < M12 < M48; $P < 0.05$). Also, the ability of Zn to induce iMT increased proportionally with the progression of the idiorrhythms from I = Mx/1 to 8Mx/8 ($P < 0.001$). When rats were fed M3, less Zn was required to induce iMT than when they were fed M12 or M48. Thus, within the M and E limits of this study, the better the nutritional Zn status of the animal, the more Zn is required to induce iMT and vice versa. The fact that iMT was increased means that the amount of available Zn was not proportional with the actual steady state of its metabolism. This indicates that for any Zn supplementation program to be effective, it should progress gradually from a lower to a higher Zn dose relative to the given nutritional Zn status.

Keywords: Zinc dose-rate idiorrhythm; Structured dietary intake; Intestinal metallothionein induction; Zinc nutritional status; Zinc repletion rate; Gastrointestinal tract as a sensory organ

1. Introduction

Today, nutrition is mostly perceived as a constant intake of a dietary nutrient in equilibrium with a homoeostatically controlled metabolic steady state. However, it would be more realistic to view nutrition as a series of repetitive events including ingestion and digestion of food. This process could be regarded as the nutrient dose-rate dependent state of fluctuating steady state of metabolism [1–6]. By using an idiorrhythmic experimental feeding model, we recently demonstrated that the metabolic efficiency of dietary zinc (Zn) depends upon the dose-rate of Zn intake [2]. Later, we demonstrated that idiorrhythmic dose-rate feeding yields a different metabolic response than that elicited by the classical dose-response feeding model, which is based on a continuous intake of a diet with a consistent nutrient composition [3]. Varying the idiorrhythmic dose-rate of dietary Zn modified the metabolic efficiency of Zn by as much as 50% [4]. Further, this model provides us with the experimental means to study interactions between trace elements at the physiological level of their dietary intake [5].

One of the messages of idiorrhythmic feeding to a real-world situation is that if an individual consumes a limited amount of Zn, but not large enough to cover the daily requirements over a given length of time, it would be better to consume a generous amount of Zn from time to time than to consume a sub-optimal amount all the time [6]. Thus, the simultaneous and exact quantification and structuring of both dose (amplitude) and time (frequency) in the idiorrhythmic feeding model offers new possibilities for obtain-

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ing deeper insights into the complexity of metabolic processes in the body. It could have a distinct advantage over the more commonly used intuitive models such as meal partitioning, dilution diets, pulse, and/or intermittent feeding where the element of time is not intrinsically scrutinized. Thus, in the recent discussions about the pros and cons of intermittent vs continuous daily administration of supplemental iron [7–10], two studies compared groups that differed in the length of the epoch (I = 50/1; E = 6 days vs. I =50/1; E = 1 day) [11] or that differed in both the idiorrhythm and the length of the epoch (I = 60/1; E = 6 days vs. I = 120/7; E = 21 day) [12] [See Materials and methods section for an explanation of idiorrhythmic terms and notation]. This implies that the current studies on intermittent iron supplementation, essentially a dose-rate endeavor, are arbitrary in the dosing schedule, in the total dose consumed, in the length of supplementation, and in the choice of relevant physical and/or biological end points. Translated into idiorrhythmic terms, this means that a meaningful conceptual framework is missing for comparison of the time-equivalent modulos, dose-rate idiorrhythms, and length of the idiorrhythmic epochs. Indeed, the idiorrhythmic dose-rate experimental model already includes the classical dose-response model as a special and most simple case [3].

The main objective of this study was to investigate how the induction of iMT depends upon the dose-rate idiorrhythm frequency and the dose-time equivalent modulo amplitude. In other words, how the induction of iMT depends on the variability of dietary zinc intake. In a previous experiment, we studied the induction of intestinal metallothionein (iMT) by idiorrhythmic feeding of dose-time equivalent modulos (Mx) of 12 (M12) and 24 (M24) mg Zn/kg per day over one 24-d nodus (N) of an idiorrhythmic epoch (E) [3]. It should be noted that the dose-time equivalent modulo is in effect the known descriptor of nutritional Zn status of an animal and hence its metabolic pool of Zn [13,14]. Within that modulo window, idiorrhythmic feeding did not affect the capability of iMT to be induced. This suggests the intriguing possibility that suitable idiorrhythmic spacings of Zn doses could moderate or bypass the induction of iMT, and thus presumably allow for greater Zn absorption into the body [15]. Therefore, the aim of the present study was to determine the capability of different modulos of Zn over a 48-d (two nodi) idiorrhythmic epoch to affect the induction of iMT.

2. Materials and methods

2.1. Idiorrhythmic dose-rate experimental feeding design

Idiorrhythm (I) describes a distinctly proportional and regularly recurrent pattern. Idiorrhythmic feeding designs involve offering a diet with *n* times the overall nutrient concentration (modulo, M) only every *n*th day with a diet low in that nutrient offered on all other days. As an example for Zn, an idiorrhythmic approach requires that the average daily dietary Zn concentration (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 to compensate for those days that the Zn-supplemented diet is not fed. Thus, for a protocol that involves feeding a Zn-supplemented diet every third day, the diet would contain three times the average Zn concentration and the idiorrhythm would be designated 3M/3 (Fig. 1) [2]. The conventional dose-response model, in which the nutrient is fed daily at a constant dose, could be regarded as a special case of an idiorrhythm where the time base is only one day.

In a formal sense, the relationship between the dose-rate idiorrhythms, the selected dose-time equivalent modulo level (Mx; x = mg Zn/kg per d), and the sequential number of the day on which the peak dose is administered, i.e., dosing day ($d_{n\text{th}}$), can be expressed as $I = [d_{n\text{th}}(Mx)]/d_{n\text{th}}$. For example, 6M12/6 (or I = 72/6) denotes that 72 mg Zn/kg were fed every 6 days. Thus, the larger the span between dosing days, the higher the idiorrhythm. All idiorrhythms that share the same Zn dosing day ($d_{n\text{th}}$) 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 to I (I_{Mx}). The lowest common denominator for the idiorrhythms Mx/1 to 8Mx/8 (except 5Mx/5 and 7Mx/7) is 24 days, and such a period within the idiorrhythmic epoch is called a nodus (N). In this experiment, we studied the effects of Zn on the induction of iMT over two such idiorrhythmic nodi within the idiorrhythmic epoch of 48 d. The length of any idiorrhythmic epoch is the sum of the number of inclusive nodi.

2.2. Experimental diets

The composition of the basal Zn-deficient diet (basal diet) was similar to that published by Momčilović and Reeves [4]. It was an egg-white based diet with supplemental biotin. Zinc carbonate and all the other mineral supplements were reagent grade (J.T. Baker, Phillipsburg, NY, USA, and Pflatz and Bauer, Watersbury, CT, USA).[1] The basal diet closely resembled the AIN93G diet for growing rats except that the mineral mix was reformulated to meet the requirements for phosphorus when egg white is used as the source of protein [16,17]. The basal diet contained less than 0.6 mg Zn/kg as assessed by inductively coupled argon plasma atomic emission spectrometry (ICAP-AES) [18]. 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. All analyses were within ±5% of the range given for each standard.

Three representative dose-time equivalent modulos of Zn were chosen to run over a two nodi (48 d) idiorrhythms (Fig.

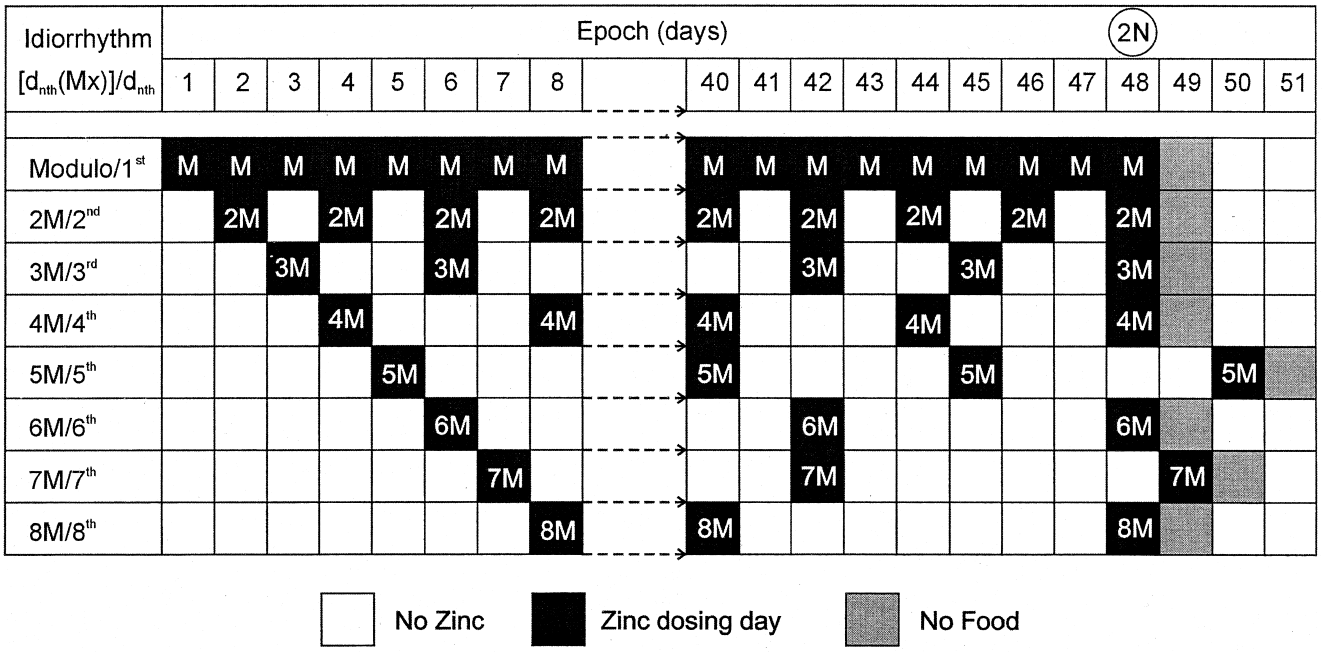

Fig. 1. Idiorrhythmic feeding experimental design. Idiorrhythm (I; dose-rate); I = $d_{nth}(Mx)]/d_{nth}$ = mg Zn/kg/d_{nth}, dnth = sequential number of Zn dosing days. Epoch (E) = 48 day period. Nodus (E) = 24 day period. Modulo (Mx; dose-time equivalent) = mg Zn/kg/d or mg of Zn/kg/E. M3 = 3 mg Zn/kg/d or 72 mg Zn/E, I = 3/1, 6/2, 9/3, 12/4, 15/5, 18/6, 21/7, and 24/8. M12 = 12 mg Zn/kg/d or 288 mg Zn/kg/E, I = 12/1, 24/2, 36/3, 48/4, 60/5, 72/6, 84/7, and 96/8. M48 = 48 mg Zn/kg/d or 1,152 mg Zn/kg/E, I = 48/1, 96/2, 144/3, 192/4, 240/5, 288/6, 336/7, and 384/8.

1). The three modulos defining the nutritional status were, low-Zn (M3; 3 mg Zn/kg per d), adequate-Zn (M12; 12 mg Zn/kg per d), and high-Zn (M48; 48 mg Zn/kg per d). Each modulo had eight analogous dose-rate idiorrhythms arranged in increasing order: (a) I = Mx/1: 3, 12, or 48 mg Zn/kg per d_1 fed daily, (b) I = 2Mx/2: 6, 24 or 96 mg Zn/kg per d_2 fed every other day and separated by a day of feeding the basal diet, (c) I = 3Mx/3; 9, 36, or 144 mg Zn/kg per d_3 fed every third day and separated by 2 d of feeding the basal diet, (d) I = 4Mx/4; 12, 48, or 192 mg Zn/kg per d_4 fed every fourth day and separated by 3 d of feeding the basal diet, (e) I = 5Mx/5; 15, 60, or 240 mg Zn/kg per d_5 fed every fifth day and separated by 4 d of feeding the basal diet, (f) I = 6Mx/6; 18, 72, and 288 mg Zn/kg per d_6 fed every sixth day and separated by 5 d of feeding the basal diet, (g) I = 7Mx/7; 21, 84, or 336 mg Zn/kg per d_7 fed every seventh day and separated by 6 d of feeding the basal diet, and (h) I = 8Mx/8; 24, 96, or 384 mg Zn/kg per d_8 fed every eighth day and separated by 7 d of feeding the basal diet. The expected *vs.* analyzed Zn contents of all 24 experimental diets are shown in Table 1. Each dose-time equivalent modulo was comprised of eight analogous Zn

Table 1
Expected vs. analyzed values for Zn concentration in experimental diets*

Idiorrhythm† (mg Zn/kg per d_{nth})	†Modulo (mg Zn kg/ per d_1)					
	Low Zn (M3)		Adequate Zn (M12)		High Zn (M48)	
	Expected	Analyzed	Expected	Analyzed	Expected	Analyzed
M/1	3	2.9 ± 0.01	12¶	12.1 ± 0.07	48‡	48.6 ± 0.04
2M/2	6	6.3 ± 0.07	24¶	23.4 ± 0.10	96‡	102.7 ± 1.56
3M/3	9	9.0 ± 0.01	36	36.4 ± 0.30	144	147.4 ± 0.49
4M/4	12¶	12.1 ± 0.07	48‡	48.6 ± 0.04	192	198.7 ± 1.30
5M/5	15	15.1 ± 0.04	60	60.6 ± 0.60	240	248.1 ± 1.87
6M/6	18	18.1 ± 0.07	72	74.4 ± 0.46	288	312.8 ± 4.45
7M/7	21	20.9 ± 0.07	84	85.2 ± 0.81	336	357.8 ± 1.12
8M/8	24¶	23.4 ± 0.10	96‡	102.7 ± 1.56	384	412.1 ± 5.90

* Values are means ± SEM of mg/kg diet, $n = 3$.

† For details of terms and procedures see Fig. 1 and the idiorrhythmic dose-rate experimental feeding design in Methods. Basal zinc deficient diet contained 0.5 ± 0.07 mg Zn/per kg ($n = 12$).

¶ Identical for M3 and M12.

‡ Identical for M12 and M48.

dose-rate idiorrhythms, and each was run as an independent experiment. The Zn requirement proposed by the National Research Council [19] is 12 mg Zn/kg diet for growing rats and is equal to that of Zn dose-rate $I_{M12} = 12/1$ (12 mg Zn/kg per d). Therefore, $I_{M12} = 12/1$ was the control idiorrhythm. Each modulo was run as an independent experiment and, therefore, had a control idiorrhythm of its own to check for possible time effects.

2.3. Experimental animals

The study was approved by the Animal Use Committee of the U.S. Department of Agriculture, Agriculture Research Service, Grand Forks Human Nutrition Research Center and was in accordance with the guidelines of the National Research Council on the experimental use of laboratory animals [20].

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 dark-light cycle. They were given free access to powdered experimental diets, and deionized water (Super Q System, Millipore Corp., Bedford, MA, USA). All rats except those in modulos 5M/5th and modulos 7M/7th were fed their respective diets for 48 d; 5M/5th rats were fed for 50 d and 7M/7th rats were fed for 49 days (Fig. 1). At the end of the feeding periods, the rats were deprived of food overnight (16 h) and killed by exsanguination from the abdominal aorta after halothane inhalation. Immediately after the rats died, a 20 cm length of the small intestine (regardless of the size of the animal), starting at the pylorus, was removed, the lumen contents washed out with ice-cold saline, and the mucosal lining removed by lightly scraping with the edge of a glass slide. Gastrointestinal (GIT) mucosal scrapings were weighed to the nearest mg and then kept frozen at −80° C until randomly analyzed for iMT over a 7-day period.

2.4. Intestinal metallothionein (iMT) by the cadmium-hemoglobin displacement assay

The mucosa were homogenized and prepared for iMT analysis by the method of Eaton and Cherian [21], except that a portion of the homogenate was heated for 5 min at 95° and then centrifuged for 5 min at 10,000 g. The supernatant was used for iMT analysis. The Tris buffer contained one mmol of 2-mercaptoethanol/L, pH 7.4. The amount of iMT in the mucosa was expressed as Cd binding potential, and was 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 6.5 kDa [21,22]. To control for between experiment variability, values were normalized by expressing them as percentages of the average value of iMT obtained from rats fed the control idiorrhythm, $I_{M12} = 12/1$.

2.5. Statistical analysis

Results were expressed as means ± SEM for seven or eight rats per group. All differences were considered significant if $P \leq 0.05$. The effect of three different dietary Zn dose-time equivalent modulos, each with eight analogous Zn dose-rate idiorrhythms on iMT induction was assessed by two-way ANOVA. The Ryan-Einot-Gabriel-Welsh (REGW) multiple F test was used to determine if the mean values between idiorrhythms and modulos were significantly different [23]. The individual difference between all the idiorrhythms were assessed with Tukey's standardized range test [24]. The induction potential of Zn on iMT was quantitatively assessed by a slope-ratio assay of the M3, M12, and M48 analogous idiorrhythms. Šidak contrasts were used for pair-wise comparisons of the slopes of the analogous Zn dose-rate idiorrhythms [24].

3. Results

The weight of the GIT mucosa was higher in low-Zn (M3) animals than in those fed adequate-Zn (M12) and high-Zn (M48) diets ($M3 < M12 = M48$; $P < 0.05$ for M3 vs. M12 and M48) (Table 2). Animals fed M3 diets had considerably lower body weights (data not shown) but proportionally much heavier GIT mucosa than those fed M12 and M48 idiorrhythmic diets. There was no difference in GIT mucosa weight between the M12 and M48 idiorrhythms. The GIT mucosa weights also depended upon the idiorrhythm so that they were generally higher with an increase in the idiorrhythm within all three modulos. However, that increase showed a complex bimodal pattern; we could identify two peaks at $I = 9/3$ and 21/7 for M3, another two peaks for M12 at $I = 48/4$ and $I = 96/8$, but only one peak for M48 at $I = 384/8$. It should be noted that because the body weight and GIT mucosa weights of idiorrhythmically fed animals did not change linearly with a change in the idiorrhythm, a strong $M \times I$ interaction was present.

Idiorrhythmic dose-rate feeding of Zn induced proportional increases in iMT with both the dose-time equivalent modulo and from low to high idiorrhythms (Table 3). The data show that the higher the Zn modulo the greater the capacity for iMT to be induced. The peak iMT values were reached on days 8, 7, and 6 for M3, M12, and M48 idiorrhythms, respectively—the higher the dose the earlier the peak.

The presence of a strong $M \times I$ interaction suggests that considerable complexity was associated with varying the idiorrhythmic Zn dose-rate. To facilitate the cross-comparison between various idiorrhythms and to visualize the possible qualitative traits describing the difference between them, we integrated the graphic boxes (instead of superscripts) into Table 3 showing the pattern of the statistical profile. Thus, visual examination of the boxes showed that within each modulo the capacity of Zn to induce iMT

Table 2
Effect of zinc dose-rate idiorrhythm (I) and zinc dose-time equivalent modulo (M) on the weight of the intestinal mucosa expressed as g/100 g body weight

Idiorrhythm* (mg Zn/kg per d_{nth})	*Modulo (mg Zn kg/ per d_1)					
	M3, Low Zn^A		M12, Adequate Zn^B		M48, High Zn^B	
	I	Mean ± SD	I	Mean ± SD	I	Mean ± SD
$M/1^{AB}$	3/1	1.90 ± 0.12^{bdg}	$12/1^{\dagger}$	1.00 ± 0.06^{a}	48/1	1.24 ± 0.10^{ace}
$2M/2^{A}$	6/2	1.93 ± 0.12^{bdfg}	24/2	0.96 ± 0.06^{a}	96/2	1.08 ± 0.03^{ac}
$3M/3^{CD}$	9/3	2.85 ± 0.21^{h}	36/3	0.97 ± 0.08^{a}	144/3	1.16 ± 0.04^{ac}
$4M/4^{BCD}$	12/4	2.34 ± 0.18^{bdfh}	48/4	1.53 ± 0.13^{aceg}	192/4	1.03 ± 0.04^{ac}
$5M/5^{ABC}$	15/5	1.84 ± 0.12^{beg}	60/5	1.21 ± 0.09^{ac}	240/5	1.16 ± 0.09^{ac}
$6M/6^{ABCD}$	18/6	2.39 ± 0.40^{dfh}	72/6	1.11 ± 0.10^{ac}	288/6	1.29 ± 0.06^{ace}
$7M/7^{CD}$	21/7	2.51 ± 0.13^{fh}	84/7	1.37 ± 0.04^{aceg}	336/7	1.21 ± 0.09^{ac}
$8M/8^{D}$	24/8	2.43 ± 0.25^{fh}	96/8	1.49 ± 0.04^{aceg}	384/8	1.69 ± 0.16^{bceg}

Means ± SD, $n = 8$. (For details of terms and procedures see Fig. 1 and Idiorrhythmic dose-rate experimental feeding design).

^{AB} Two way ANOVA: M, $P < 0.01$; I, $P < 0.01$; and M × I interaction, ($P < 0.05$). M and I values bearing different upper case superscript were significantly different ($P < 0.05$) by the REGW multiple F test [15].

^{a,b,c,d,e,f,g,h} Compared within and between the modulos, mean values not sharing a common lower case superscript letter were significantly different, $P < 0.05$ (REGW multiple F test).

† Control idiorrhythm (I = M12/1).

tended to rise with an increase in idiorrhythm. Indeed, when the idiorrhythmic dosing was $I_{M3} = 24/8$ (low-Zn, M3; I = 24 mg Zn per kg diet on every 8th day), iMT values did not differ from that of $I_{M3} = 3/1$. However, whereas the former, 24/8, also did not differ from $I_{M48} = 384/8$ ($P > 0.05$), the latter, 3/1 was only as high as $I_{M48} = 144/3$ ($P > 0.05$). Both idiorrhythms had their distinguishing statistical profile in how much they did or did not differ from the other idiorrhythms. The observed qualitative aspect of the data pattern leads us to conclude that the iMT inductive capacity of idiorrhythmic Zn within the modulo depends proportionally upon the increase in the amount of Zn administered on the idiorrhythmic Zn dosing day. Thus, we infer that the lower the dose-time equivalent modulo the lower the amount of idiorrhythmically administered Zn that will be needed to induce iMT. The amount of zinc needed to induce iMT for M3, M12, and M48 was associated with idiorrhythms $I_{M3} = 24/8$, $I_{M12} = 72/6$, and $I_{M48} = 192/4$, respectively.

We also analysed the modulo effect with a slope-ratio assay and found that the slope for M3 idiorrhythms was less than that for M12 and M48 idiorrhythms ($P < 0.01$ for both comparisons); whereas, the slopes for M12 and M48 idiorrhythms did not differ ($P < 0.22$), although the slope for M48 idiorrhythms was steeper (Fig. 2). It is evident that the two-way ANOVA was a more powerful discriminatory test having a greater resolving power than the slope-ratio assay for assessing the difference between these two modulos. As already shown, low-Zn M3 idiorrhythms had the highest GIT mucosa weight but also had the lowest iMT values as compared with those of adequate-Zn M12 and high-Zn M48 idiorrhythms.

The ability of Zn to induce iMT generally increased with the progression of the idiorrhythms from I = Mx/1 to I = 8Mx/8 ($P < 0.001$). The significant M × I interaction takes precedence over the main effect in the ANOVA. For M3, idiorrhythms did not increase iMT until I was greater or equal to 7. For M12 there was no increase until I was greater or equal to 5. In M48, iMT increased linearly from I = 1 through I = 6, but then the amount of iMT decreased when I = 7 or 8. Thus, over the wide range of the peak doses of dietary Zn (3–384 mg/kg), less Zn was needed to induce iMT when rats were fed M3 idiorrhythms then when they were fed M12 and even the more so when they were fed M48 idiorrhythms.

Analogous idiorrhythms (the idiorrhythms of the same time base, but belonging to different Mx) can in theory be referred to as the "dosing of time" where the time component of the abscissa of a given idiorrhythm is an independent variable and the ordinate of the iMT response across the different Mx is a dependent variable. Thus, each set of analogous idiorrhythms can be analyzed as an independent dose-response set that 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 effects of variability in dietary Zn allows us to treat each of the analogous idiorrhythms as if it were a different source of Zn whose metabolic availability we would like to compare [3].

Accordingly, we compared the dependency of iMT induction across M3, M12, and M48 on any given idiorrhythm from I = Mx/1 to I = 8Mx/8 (Fig. 3). These results showed that the capacity of Zn to induce iMT depends on the time component of the idiorrhythm. Indeed, feeding rats diets containing 72, 84, and 96 mg Zn/kg every 6th, 7th, and 8th day, respectively, was sufficient to induce significant increases in iMT even though their average intake per day for the duration of the study would be equivalent to consuming a diet containing 12 mg Zn/kg continuously. The statistical differences between the slopes of analogous idi-

Table 3
Effect of zinc dose-rate idiorrhythm (I) and zinc dose-time equivalent modulo (M) upon the induction of intestinal metallothionein, expressed as a percentage of control iMT, over a two nodi (48d) idiorrhythmic epoch*

Modulo† (mg Zn kg/per d_1)	Idiorrhythm† (mg Zn kg per d_{nth})	iMT‡ [Mean (1 SD Range)]	Difference¶
M3 Low Zinc			
	3/1	0.96 (0.77–1.21)	■■■■■■□□□□□
	6/2	1.00 (0.89–1.13)	■■■■■■□□□□□
	9/3	0.81 (0.63–1.04)	■■■■■□□□□□□
	12/4	0.95 (0.79–1.14)	■■■■■□□□□□□
	15/5	1.04 (0.88–1.25)	■■■■■■□□□□□
	18/6	1.39 (0.78–2.49)	■■■■□□□□□□□
	21/7	1.16 (0.99–1.35)	■■■■■■■□□□□
	24/8	1.36 (1.03–1.79)	■■■■■■■■□□□
M12 Adequate Zinc			
	12/1§	0.99 (0.84–1.17)	■■■■■■□□□□□
	24/2	1.03 (0.88–1.21)	■■■■■■□□□□□
	36/3	1.18 (0.92–1.51)	■■■■■■■□□□□
	48/4	1.12 (0.83–1.52)	■■■■■■■□□□□
	60/5	1.45 (1.21–1.73)	■■■■■■■■■■□
	72/6	1.73 (1.45–2.07)	□■■■■■■■■■■
	84/7	2.39 (1.91–2.99)	□□■■■■■■■■■
	96/8	2.11 (1.41–3.17)	□□■■■■■■■■■
M48 High Zinc			
	48/1	0.91 (0.56–1.47)	■■□■■■□□□□□
	96/2	1.19 (0.78–1.83)	■■■■■■■□□□□
	144/3	1.72 (1.01–2.93)	□■■■■■■■■□□
	192/4	2.41 (1.52–3.82)	□□■■■■■■■■■
	240/5	2.74 (1.66–4.55)	□□■■■■■■■■■
	288/6	4.00 (2.64–6.06)	□□□□□□■■■■■
	336/7	3.80 (2.40–6.02)	□□□□□□■■■■■
	384/8	3.14 (1.59–6.21)	□□□□□□■■■■■

* Seven to eight animals per idiorrhythm.

† For details of terms and procedures see Fig. 1 and Idiorrhythmic dose-rate experimental feeding design in Methods. Two way ANOVA: M, $P < 0.0001$; I, $P < 0.0001$; M × I interaction, $P < 0.0001$; M3 < M12 < M48 ($P < 0.01$ for every comparison).

‡ Because the data for these parameters did not follow a normal distribution, a natural logarithm transformation was performed before the ANOVA was run. These values represent the back transformed means plus 1 SD range.

¶ Compared within and across modulos, mean values not sharing a common filled box (■) were significantly different, $P < 0.05$ (Tukey's Studentized range (HSD) test).

§ Control idiorrhythm.

orrhythms are shown in Table 4. The results clearly show that iMT induction depends considerably on the quantity and duration in dietary Zn supply from the same food source.

4. Discussion

The effect of idiorrhythmic Zn dose-rate feeding on both the weight of gastrointestinal mucosa and the capacity of Zn to induce iMT was different from that of animals continuously fed Zn (I = Mx/1). We observed that the nutritional Zn status dependent on dose-time equivalent modulo was inversely related to the mass of the GIT mucosa. Paradoxically, the mass of the gastrointestinal mucosa of the low-Zn idiorrhythmically fed animals was higher than that of the animals fed either adequate-Zn, or those fed the high-Zn idiorrhythmic diets. This indicates that most of the available Zn in Zn-deficient animals is used to maintain the functional integrity of the GIT before the remainder of Zn can be distributed to other tissues of the body and subsequently used for growth and maintenance. Alternatively, it is possible that Zn deficiency produced hypertrophy in the GIT mucosa similar to parakeratosis, and impaired cell capacity to differentiate [25,26]. Initially we thought that such a cellular overgrowth may be related to the changes in the rate of apoptosis, which apparently is increased in the lymphatic tissue of zinc deficient rats [27,28], but no report was available on the apoptosis of GIT mucosa cells in the Zn-deficient rats.

Recently, McBurney [29] emphasized that the GIT is not a simple tube, but a vital organ with essential functions for the digestion and absorption of food, for selective exclusion of bacteria and toxins from the body, and for the generation of immunological defences. He pointed out that although it has been assumed that the gut and other tissues "rest" during starvation, recent evidence suggests that the gut is not quiescent. In the absence of oral intake, nutrients required by the mucosa must be obtained from the serosal side of the gut lining, and homeostasis must be established with the liver,

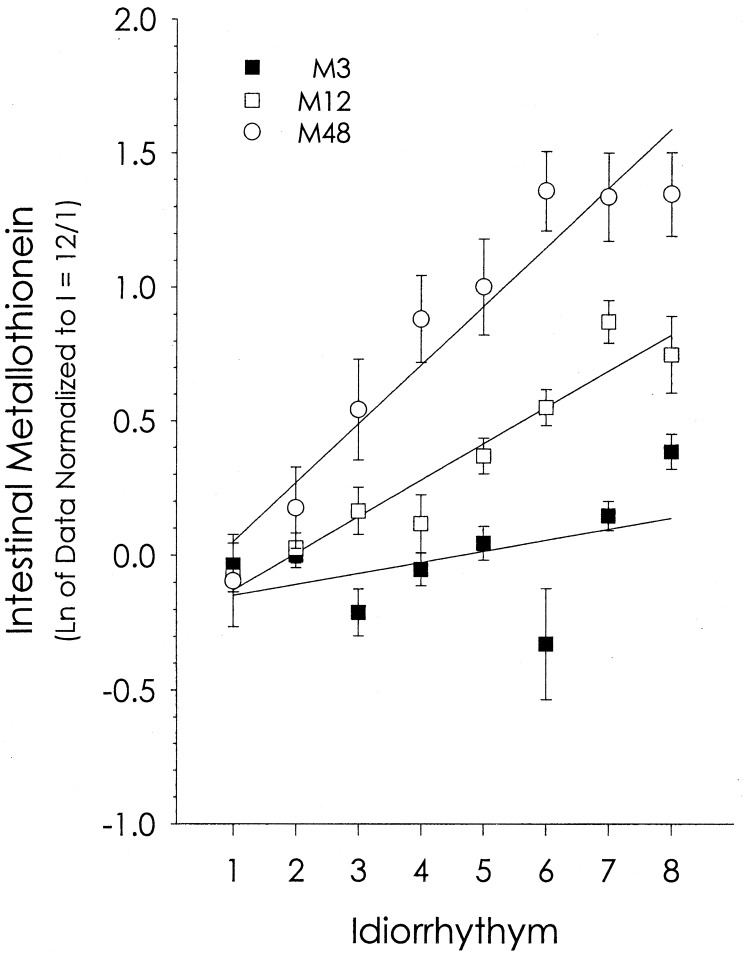

Fig. 2. Slope-ratio assay of the effects of Zn dose-time equivalent modulos M3, M12, and M48 upon intestinal metallothionein (iMT) of male weanling rats. Values are means ± SEM, $n = 8$. a and b represent the constants of the general equation:

$$\ln Y = a \ln X + b.$$

$$\ln Y_{M3} = 0.034 \ln X + -0.168 \; (r^2 = 0.058); \text{ Slope different from 0, } p < 0.06$$

$$\ln Y_{M12} = 0.128 \ln X + -0.246 \; (r^2 = 0.58); \text{ Slope different from 0, } p < 0.001$$

$$\ln Y_{M48} = 0.164 \ln X + 0.074 \; (r^2 = 0.35); \text{ Slope different from 0, } p < 0.001$$

Slope M3 is different from M12 and M48 ($p < 0.002$), but M12 and M48 do not differ.
See legend on Fig. 1 for description of I and M.

muscle, and other metabolically active cells. Thus, the central role of the gut in determining the nutrient requirement of the whole-organism is a function of the whole gut mass, which reflects the number and metabolic activity of its constituent cells.

Selectivity of metal ion transport across the intestinal epithelium is achieved by specific transporters that are under genetic and metabolic control [30,31]. Compared to feeding an extremely Zn-deficient diet, fasting, and/or starvation, the low-Zn modulo diet may provide enough Zn to maintain the physiological integrity of the intestinal mucosa, but the metabolic performance of cells may be different from that of a well-fed state [32]. Indeed, cells generally respond to stimulation by increased enzyme production in an all-or-none fashion instead of responding in a gradual, stoichiometric, manner [33]. It remains to be investigated whether such a first-come, first-served partitional "piracy" occurs between the cells of the GIT mucosa and those of the rest of the body of Zn-deficient animals.

We intuitively expected that the higher the Zn dose-time equivalent modulo, the better the nutritional status of Zn, the larger the Zn metabolic pool, and the greater the inductive capacity to generate iMT. However, the results showed that the inductive capacity of iMT by Zn increased with the progression of the idiorrhythms within each individual modulo sequence. In other words, when rats were fed the low-Zn modulo, less Zn was required to induce iMT synthesis than when they were fed adequate- and/or high-Zn modulos. In a

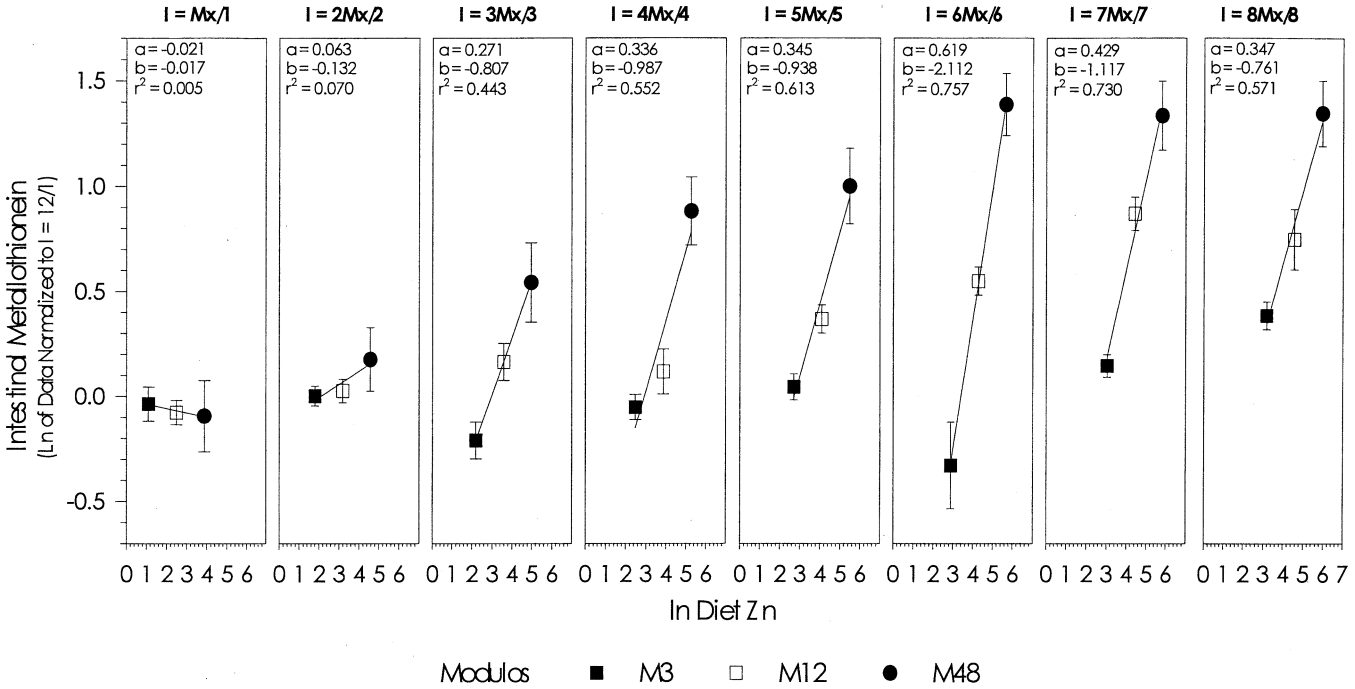

Fig. 3. Slope-ratio assay analysis of the effect of analogous Zn dose-rate idiorrhythms upon intestinal metallothionein (iMT) of male weanling rats. Each block represents the set of M3, M12, and M48 analogous idiorrhythms: I = Mx/1; I = 3/1, 12/1, and 48/1; I = 2Mx/2 where I = 6/2, 24/2, and 96/2; I = 3Mx/3 where I = 9/3, 36/3, and 144/3; I = 4Mx/4 where I = 12/4, 48/4, and 192/4; I = 5Mx/5 where I = 15/5, 60/5, and 24/5; I = 6Mx/6 where I = 18/6, 72/6, and 288/6; I = 7Mx/7 where I = 21/7, 84/7, and 336/7; I = 8Mx/8 where I = 24/8, 96/8, and 384/8. Values are mean ± SEM, $n = 8$. a and b represent the constants of the general equation: $\ln Y = a \ln X + b$. See legend on Fig. 1 for descriptions of I and M.

previous experiment we observed no difference in iMT induction capacity of an adequate-Zn (M12) and an ample-Zn (M24) idiorrhythms over a 24-day idiorrhythmic epoch [3]. The larger modulo window and a longer idiorrhythmic epoch, as used in this experiment, allowed us to detect the gradual dependence of iMT induction on the increase in the Zn dose-time equivalent modulo. Whereas the ratio between M12 and M24 in the previous experiment was 1:2, the ratio between M3, M12, and M48 in this experiment was 1:4:16; thus covering a much larger span of idiorrhythmic Zn dose-rate concentrations.

In comparison to the control I = 12/1, none of the continuously (daily) Zn-fed animals (I = Mx/1) increased iMT induction after 48 days following the introduction of idiorrhythmic feeding, regardless of the modulo level. Reeves [22] reported that if two groups of animals were fed either 50 or 350 mg/Zn kg, corresponding to the continuous daily idiorrhythms I = 50/1 and 350/1, and with an arbitrary epoch of 30 days, iMT concentrations would reach a peak at 10 days (20 *vs.* 100 μmol iMT/kg) and by 30 days it was back down to 17 vs. 30 μmol/kg. Thus, in continuously fed animals, the dose dependent iMT induction was a transitory phenomenon. Reeves [22] showed in the same paper that when the amount of Zn was increased stepwise by 50 mg/kg each week in a cascade from 50 to 100, 150, 200, 250, 300, and 350 for six weeks, there was no effect on iMT induction. Apparently, iMT induction was adequately tuned to the pace of a gradual change in dietary Zn and did not show spikes characteristic of metabolic steady state disequilibria. This indicates that at every Zn intake different from the previous one, there is a time lapse before the metabolic machinery is "tuned up" to meet the new circumstances of different nutrient supply. Such tachyphylaxic resistance may be used in assessing the rate at which a new steady state of a Zn metabolic pool is achieved (tachyphylaxis is defined as a decrease in a biological response to a stimulus of constant strength, notably of excessive or pharmacologic amounts) [34].

In contrast to the continuous daily feeding of the same

Table 4
Šidák contrast-P values for data shown in Fig. 3. Pairwise comparison of the slope of analogous zinc dose-rate idiorrhythms for zinc dose-time equivalent M3, M12, and M48

Analogous Idiorrhythms	Analogous Idiorrhythms						
	2Mx/2	3Mx/3	4Mx/4	5Mx/5	6Mx/6	7Mx/7	8Mx/8
Mx/1	NS*	NS	<0.05	<0.05	<0.05	<0.05	<0.05
2Mx/2		NS	NS	<0.05	<0.05	<0.05	NS
3Mx/3			NS	NS	NS	NS	NS
4Mx/4				NS	NS	NS	NS
5Mx/5					NS	NS	NS
6Mx/6						NS	NS
7Mx/7							NS

* NS, not significantly different; $p < 0.05$, significantly different.

In contrast to the continuous daily feeding of the same

dose of Zn, or increasing it gradually to allow for metabolic tuning, the capacity of Zn to induce iMT within the dose-time equivalent modulo depended upon the idiorrhythm in that only the higher order idiorrhythms ($\geq$6Mx/6) will appreciable induce iMT. This trend is more apparent with the slope-ratio data analysis (Table 4, Figs. 1 & 2) than with the REGW analysis (Table 3). Also, with low-Zn intake, as little as 24 mg Zn/kg diet/d_8 induced iMT, albeit statistically non-significant. Whereas, when rats were fed a high-Zn intake, it required almost 300 mg Zn/kg diet/d_6 to induce iMT. Our observation that the capacity of Zn to induce iMT depends upon the underlying nutritional Zn status of the animal, i.e., that more Zn is needed to induce iMT when Zn status is high than when it is low, is in accord with Weber's observation that the increment in stimulation required to produce a barely noticeable difference between two stimuli was proportional to the size of the stimuli [35,36] Apparently the GIT behaves as if it were a sensory organ that processes the molecular stimuli coming from the digestion of nutrients [3].

Can we bypass the iMT induction in the GIT mucosa by spacing dietary Zn intake? The answer to this question is an elusive, oblique, and situational one. Within the modulo and epoch limits of this experiment, the better the nutritional Zn status of the animal, the more Zn that is required to induce iMT and vice versa. Thus, the amount of Zn that will be tolerated without inducing an increase in iMT depends upon the size of the underlying Zn metabolic pool, i.e., the given nutritional status [13,14], the two conditions that are themselves subject to change. Hence, adequate idiorrhythmic coupling of Zn dose to the frequency [37] may either induce or bypass iMT induction depending upon the particular dose-time equivalent modulo and dose-rate idiorrhythm. Their interplay at any given moment would result in a unique steady state of some Zn metabolic pool. Such a steady state is only a special case of dynamic equilibrium between the metabolic pathways within the system of the body that will be further tuned up or down depending upon the actual changes in the nutritional environment [35]. The mere fact that iMT is increased means only that the amount of available Zn is not at pace with the actual steady state of metabolism. Apparently, the role of iMT is to serve as a "fuse" in a metabolic circuitry of the body by helping it to stand up to the sudden "surges" in dietary Zn and to allow it to tune up or down from one metabolic steady state to another.

Dietary Zn intake and Zn nutritional status of humans differ considerably depending on the socio-economical conditions. Jackson et al. [38] found that plasma Zn and Zn balance of lactating women of low socio-economical status in Brazil adapted to daily Zn intakes as low as 6 mg/d (range 5.8 to 11 mg/d). This intake is lower than would be found with Western diets of 15 mg or more/d [39,40]. Therefore, less Zn might be needed to induce iMT in the former than in the latter, i.e., that the induction of iMT depends on the nutritional status of the individual. Any extra intake of Zn, on a limited basis, might initiate iMT synthesis and affect the partitioning of available Zn for defensive purposes rather than for optimal management of limited resources. Thus a program to supplement Zn to a population with low-Zn status would be more efficient if—depending upon Zn nutritional status—the supplementations were introduced in small increments over time rather than large ones abruptly. We think that such reasoning may apply to the targeted use of any other trace element or vitamin, especially if specific dynamic indicators of metabolic steady state are already available (e.g. mucosal ferritin) [41,42]. The observed principles shown here with the idiorrhythmic induction of iMT with Zn may also help us to better understand the pathological physiology of re-feeding practices following famine and starvation. Then perhaps we could develop not only clinical approaches [43] but also more scientific based approaches to those problems.

The observation that the capacity of Zn to induce iMT may depend upon Zn status may offer some new clues as to why children with protein energy malnutrition perform much better if they are gradually introduced to a normal diet rather than all at once [43]. It may be because they have a decreased capacity to handle the sudden increase in nutrient load and the system begins to react defensively.

Notes

Mention of a trademark or proprietary product does not constitute a guarantee of warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that also may be suitable.

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