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Alterations in lipid membrane fluidity and the physical state of cell-surface sialic acid in zinc-deficient rat erythrocyte ghosts

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Erythrocyte ghosts, prepared from the blood of rats fed zinc-deficient diets, were evaluated for membrane fluidity and surface sialic acid properties using spin-labeled probes and electron spin resonance (ESR) spectroscopy. These physical parameters of the erythrocyte ghosts from the zinc-deficient group were compared to those for erythrocyte ghosts obtained from ad libitum and pair fed controls consuming zinc-adequate diets. As the animals became progressively zinc deficient, the erythrocyte ghost membranes became more fluid than those from the control groups. In addition, the apparent rotational correlation time of Tempamine spin probes on surface sialic acid residues was smaller for the zinc deficient group, indicative of an increased rotational mobility of the spin label. These results suggest that zinc deficiency can have pronounced effects on the physical state of membrane bilayer lipids and cell surface carbohydrates and supports the view that many of the pathological signs of zinc deficiency are due to a general membrane defect.

Zinc is an essential trace element for man and animals. It is involved in over 200 metalloenzymes, is required for normal nucleic acid and protein metabolism, and is necessary for the proper maintenance and stabilization of biological membranes [1]. Zinc deficiency in animals is characterized by anorexia, growth retardation, skin lesions, altered protein metabolism, impaired immune function, delayed wound healing, and various other pathological signs [2]. It has been suggested that many of the pathological signs of zinc deficiency can be explained by a general membrane defect [3]. Zinc salts are often added to buffers in the isolation of membrane fractions [4] and a direct stabilizing effect of zinc on erythrocyte ghosts has been demonstrated [5]. Zinc also has been shown to decrease the osmotic fragility of erythrocytes [6]. Total zinc content in erythrocytes from zinc-deficient rats was not significantly less than that in erythrocytes from zinc-adequate controls; however, a marked decrease in membrane-bound zinc was observed in the zinc-deficient animals [7]. Zinc-deficient rats have anorexia and do not respond to the administration of a variety of agents known to increase food intake [8]. We postulated that alterations in receptor

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response for factors such as catecholamines and opiates may account, at least in part, for the anorexia of zinc deficiency. Since alterations in various receptors have been demonstrated to result from changes in membrane fluidity, e.g., serotonin [9] and catecholamines [10], and receptors are often glycoproteins, this study was undertaken to determine the effect of zinc deficiency on membrane fluidity and cell-surface sialic acid using the erythrocyte ghost as a model membrane system.

Materials and Methods. Sixty male weanling (22–26-day-old) Sprague-Dawley rats (Hilltop Lab Animals, Inc., Scottdale, PA) were divided into three equal groups having similar mean weights: zinc-deficient (ZD) (65 ± 5 g), ad libitum controls (AL) (75 ± 2 g) and pair-fed controls (PF) (65 ± 5 g). Animals were fed a zinc-deficient pelleted diet (Table I) from a commercial supplier (Ziegler Brothers, Inc., Gardners, PA). Distilled and deionized water containing 30 ppm zinc was provided ad libitum to the AL and PF controls while there was no zinc supplementation in the water for the ZD group. Rats in the AL and ZD groups were allowed free access to food. Each rat in the PF group was allotted a daily ration of food equal to the amount eaten the previous day the corresponding ZD paired animal. The rats were housed in stainless steel wire-bottom cages and were maintained in an environment specifically designed for trace metal studies as previously described [11]. At weekly intervals, two animals from each group were killed by exsanguination following light pentobarbital anesthesia. Whole heparinized blood was collected for electron spin resonance (ESR) studies. Leg bones were removed for zinc analysis. Mean bone zinc concentration (μg/g) was used as an index of zinc status for each group [12]. After dry ashing, tissue zinc concentrations were determined using a Varian Model AA-375 atomic absorption spectrophotometer as previously described [13].

TABLE I

COMPOSITION OF DIET [a]

Ingredient	g/kg
Maize starch	312
Sucrose	310
Egg white solids	200
Corn oil	100
Custom salt mix [b]	40
Cellulose powder	30
Vitamin premix [c]	5
Choline chloride	3

[a] By analysis: 0.7 μg Zn/g diet with no added phytate.

[b] Bernhart and Tomarelli [22] modified zinc free salt mixture (% composition of mixture): calcium (as carbonate and phosphate) 75.6; copper (as cupric citrate) 0.558; magnesium (as oxide) 2.5; manganese (as citrate) 0.835; potassium (as iodide, dibasic phosphate and sulfate) 14.901; sodium (as chloride and phosphate) 5.2; citric acid 0.227; maize starch (in place of zinc citrate) 0.133.

[c] Vitamin mixture (g/kg of mixture): retinyl palmitate 9.24; ergocalciferol 1.604; DL-α-tocopheryl acetate 40.04; menaphthone 1.198; cyanocobalamin 1.815; thiamin mononitrate 4.00; riboflavin 8.00; nicotinamide 20.00; calcium D-pantothenate 12.00; pyridoxine hydrochloride 2.00; steroylmonoglutamic acid 0.10; D-biotin 20.00; *myo*inositol dihydrate 80.00; maize starch 800.003.

Blood from all rats was collected into heparinized tubes and within one hour erythrocyte ghosts were prepared as previously described [14]. The incorporation of the spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyl-oxyl (5-NS) noncovalently into the membrane lipid phase of the erythrocyte ghosts was accomplished using the thin-film technique after evaporation of chloroform [14]. The 5-NS-labeled erythrocyte ghosts were used to determine the effect of zinc deficiency on membrane fluidity as measured by ESR. To determine if zinc deficiency altered molecular interactions occurring at the external surfaces of the erythrocyte ghosts, a spin-label specific for sialic acid residues was employed for additional ESR measurements. The spin-label, 2,2,6,6-tetramethyl-4-aminopiperidine-1-oxyl (Tempamine), was bound covalently to sialic acid residues employing reductive amination of periodate treated membranes [15,16]. Under these conditions, no nonspecific incorporation of the spin label was observed [15,16].

Magnetic resonance measurements were obtained using a Varian E-109 X-band ESR spectrometer employing an E-238 quartz sample cell and a TM mode resonant cavity in a constant temperature and humidity room. Dry nitrogen gas was used to purge the resonant cavity and to maintain constant temperature for all samples.

Membrane lipid order and motion were as-

sessed by observing the half-width at half-height (Δh_L) of the low-field line in the ESR spectrum of 5-NS [17] as seen in Fig. 1. Mason and co-workers [17] demonstrated that Δh_L was considerably more sensitive to small changes in lipid order and motion in erythrocyte membranes than were measurements of extrema separation of lipid-specific spin label ESR spectra. This increased sensitivity results from the observation that with decreased lipid order and increased lipid motion, line broadening occurs before the onset of changes in extreme separation. Further, unlike measurements of extrema separation, Δh_L is independent of the polarity of the local microenvironment in which the paramagnetic center of the spin label is found [17]. Analogous to linewidth changes observed in chemical exchange, reorientation of the principal axis of the spin label at a suitable rate between parallel and perpendicular orientations relative to the normal to the membrane surface leads to an increased linewidth [17]. These observations in erythrocyte membranes were shown to be consistent with theoretical models [18,19] and implied a reorientation rate in or approaching the slow motion regime (10^{-7}–10^{-8} s) [17].

In the present study, an increase in linewidth, when compared to the spectrum of a rigid lattice-limit system, was used as an average measure of an increase in the exchange rate of the principal axis system of 5-NS molecules between parallel and perpendicular orientations. It is expected that such reorientation would be more facile in a lipid environment of decreased lipid packing and increased lipid motion (i.e., increased fluidity). The ESR spectrometer settings for these measurements were as follows: microwave power 16 mW, scan range 40 G, modulation amplitude 0.32 G, time constant 0.25 s, scan time 16 min. The rotational motion of spin-labeled sialic acid was inferred by calculation of the apparent rotational correlation time of Tempamine, a parameter that may be conceptualized as the time required for the spin label to rotate through an angle of one radian [14–16,20,21]. The ESR instrumental settings were identical to those for 5-NS except the time constant was 0.5 s and the scan time was 30 min.

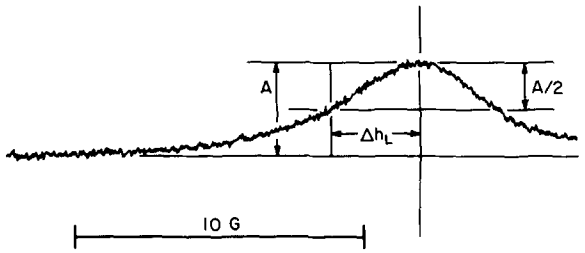

Fig. 1. Electron spin resonance (ESR) spectrum of the low-field ($M_I = +1$) line of 5-NS in rat erythrocyte ghosts.

Differences between means were tested using analysis of variance followed by *t*-tests or paired *t*-tests when appropriate.

Results and Discussion. The severe growth retardation of the zinc deficient rats in comparison to the ad libitum controls is demonstrated in Fig. 2 ($P < 0.0001$). The pair-fed controls gained more weight than the zinc-deficient animals demonstrating diminished food efficiency characteristic of the latter group [8]. Mean bone zinc concentration of all groups is shown in Table II. For all weeks of the study, the mean bone zinc concentration for the ZD group was significantly lower than that of the PF and AL groups ($P < 0.001$). By week two the mean bone zinc concentration of the PF group

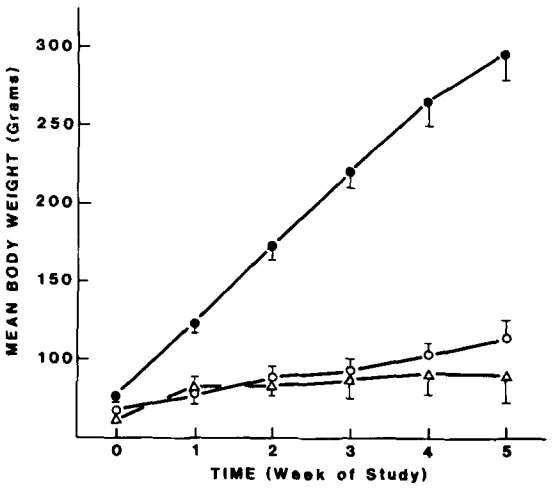

Fig. 2. Growth curves for zinc-deficient (ZD, △), pair-fed (PF, ○) and ad libitum (AL, ●) groups. The mean weight gain for the ad libitum group is significantly larger than that for the pair-fed or zinc-deficient groups ($P < 0.0001$). For each group $n = 20$ at times 0 and 1 week. At all other times n is decreased by 2 each week.

TABLE II

MEAN BONE ZINC CONCENTRATION (μg/g) OF ZINC-DEFICIENT (ZD), PAIR-FED (PF) AND AD LIBITUM (AL) RATS

Values are means $\pm$ S.D. ($n = 2$). For each week, values followed by different superscripts are significantly different for that week of the study ($P < 0.001$ for ZD vs. PF and AL; $P < 0.01$ for PF vs. AL).

Week of study	ZD	PF	AL
1	100 ± 27^{a}	211 ± 9^{b}	205 ± 22^{b}
2	66 ± 10^{a}	198 ± 8^{b}	237 ± 9^{c}
3	62 ± 3^{a}	187 ± 16^{b}	255 ± 2^{c}
4	45 ± 0^{a}	187 ± 7^{b}	265 ± 6^{c}
5	53 ± 0^{a}	190 ± 22^{b}	229 ± 10^{c}

was lower than that of the AL group ($P < 0.01$), however, no overt zinc deficiency symptoms were observed in the PF group. By the end of the fifth week, the zinc-deficient animals were experiencing many of the manifestations of the zinc deficiency such as acrodermatitis (skin lesions of zinc deficiency).

The effect of zinc deficiency on the rotational motion of the 5-NS spin label in erythrocyte ghosts obtained from these animals was measured by ESR spectroscopy (Fig. 3). The results are expressed as the ratio of the Δh_L values obtained from ad libitum or pair-fed controls to the Δh_L values obtained from zinc-deficient animals (Δh_L AL/ZD and Δh_L PF/ZD, respectively). For example, after 2 weeks the Δh_L PF/ZD ratio was approx. 1.00 indicating that the Δh_L values for the pair fed controls and the zinc deficient animals were essentially equal. However, by the fifth week, the Δh_L PF/ZD ratio had decreased to below 0.94 ($P < 0.0001$). Because the Δh_L value increases with increasing membrane lipid motion and decreased lipid order, it is clear from Fig. 3 that in progressing weeks as the animals became increasingly zinc deficient, the erythrocyte ghost membranes from the zinc deficient rats became more 'fluid' than those from pair-fed controls. The same general pattern holds true for the ad libitum controls as well.

In addition to membrane fluidity measurements, the effect of zinc deficiency on the rotational mobility of a spin label (Tempamine) covalently bound to sialic acid residues (residing on

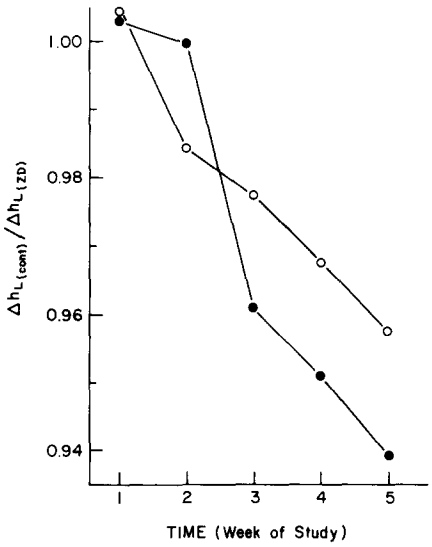

Fig. 3. Ratio of Δh_L of 5-NS in rat erythrocyte membrane ghosts obtained from controls to that in membrane ghosts obtained from zinc-deficient animals over the study weeks 1 to 5. $\Delta h_{L(cont)}/\Delta h_{L(ZD)}$ for pair-fed controls is indicated by (●), while $\Delta h_{L(cont)}/\Delta h_{L(ZD)}$ for ad libitum is indicated by (○). The linear correlation coefficients between Δh_L ratios and time were -0.93 and -0.94, respectively, ($P < 0.0001$ for each) based on a t-test with the hypothesis that there is no change in the Δh_L ratio over time, an expectation if zinc-deficiency were to have no effect or membrane lipid order and motion.

glycoproteins and glycolipids on the surface of erythrocyte ghosts [15]) was also observed. This measurement was performed on two rats from each group at week five only. The rotational mobility of the Tempamine probe covalently and specifically bound to sialic acid [15] was monitored by calculating the apparent rotational correlation time (τ_A) from the three-line Tempamine ESR spectra [20,21]. The smaller mean τ_A value for the zinc-deficient animals compared to the ad libitum and pair-fed controls (Table III) is indicative of an approx. 25% increased rotational mobility of the spin label on the external surface of erythrocyte ghosts. The simplest explanation is that zinc deficiency induces a conformational change in cell-surface glycoconjugates (perhaps as a result of increased membrane lipid motion and decreased lipid order) producing a less restricted environment for the Tempamine probe.

These magnetic resonance studies suggest that

TABLE III

APPARENT ROTATIONAL CORRELATION TIMES (τ_A) OF SPIN-LABELED ERYTHROCYTE GHOST MEMBRANES FROM ZINC-DEFICIENT (ZD), PAIR-FED (PF) AND AD LIBITUM (AL) RATS

	τ_A (10^{-10} s)	P [a]
ZD	2.60 ± 0.05	–
PF	3.48 ± 0.07	0.02
AL	3.62 ± 0.15	0.05

[a] P value calculated using a two-tailed Student's t-test with the null hypothesis that the τ_A of spin labeled sialic acid is not different than that of each control sample of erythrocyte membranes (an expectation if zinc deficiency has no effect on the physical state of this cell-surface carbohydrate).

zinc deficiency can have pronounced effects on the physical state of membrane bilayer lipids and cell-surface carbohydrates. Whether such changes account for the alterations in erythrocytes induced by zinc deficiency described above and whether certain clinical signs associated with zinc deficiency (anorexia, immune dysfunction) are the result of membrane changes in bilayer lipids and cell-surface receptors are not yet known. However, the present studies suggest that the membrane-altering effects of zinc should be examined more closely.

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