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Depressed Thymidine Kinase Activity in Zinc-Deficient Rat Embryos[1] (38995)

I. E. DREOSTI[2] AND LUCILLE S. HURLEY

Department of Nutrition, University of California, Davis, California 95616

The teratogenic consequences of a dietary zinc deficiency during pregnancy in rats are well-documented (1–4) and the suggestion has been made that the effect may arise from an impairment of DNA synthesis during embryonic development (5, 6). Recent data from studies on rat connective tissue (7) and regenerating rat liver (8) have linked the primary effect of zinc deficiency to a reduction in the activity of the zinc-dependent enzyme thymidine kinase, resulting in an overall decrease in the rate of DNA synthesis. In regenerating rat liver, the activity of thymidine kinase was shown to be significantly reduced 10 hr after partial hepatectomy in the zinc deficient rats whereas DNA polymerase, DNA synthesis and protein synthesis were affected only some hours later (8).

In an attempt to extend these findings to embryonic morphogenesis, the present study measured the activity of thymidine kinase in embryos taken from zinc deficient and control rats on Day 12 of gestation during the period of rapid organogenesis. In addition, little is known concerning the relationship of zinc and thymidine kinase, further studies were undertaken to test the effect on the enzyme of *in vitro* supplementation with zinc and a number of other divalent metal ions.

Materials and methods. Animals and diets. Virgin female rats of the Sprague–Dawley strain weighing 210 ± 10 g were bred overnight with stock colony males. On day zero of gestation as determined by the presence of sperm in the vaginal smear, the animals were placed individually in stainless steel cages and two groups were fed either the zinc deficient or control diet *ad libitum* from Day 0 to Day 12 of gestation. In addition, a group of restricted-intake animals received the control diet for the same period in amounts limited to the mean daily food intake of the deficient group. A fourth group was fed a zinc supplemented ration[3] from Day 0 to Day 9 of gestation and thereafter the zinc deficient diet to Day 12. Procedures and precautions to avoid zinc contamination were performed as described earlier (9).

The zinc deficient diet contained less than 0.5 μg zinc/g as determined by atomic absorption spectroscopy[4] and consisted of (%): isolated soybean protein,[5] 30.0; sucrose, 57.3; corn oil, 8.0; salt mix,[6] 4.0; and DL-methionine, 0.7. The soybean protein was extracted with ethylenediaminetetraacetic acid to lower its zinc content (9). The control diet was supplemented with zinc as zinc carbonate to a level of 100 μg zinc/g. In addition, all animals received vitamins[7] in glucose three times per week.

Collection of samples. On Day 12 of

[1] Supported in part by NIH research Grant No. HD-01743 from the National Institute of Child Health and Human Development.

[2] Present address: Department of Biochemistry, University of Natal, P.O. Box 375, Pietermaritzburg, South Africa.

[3] Zinc supplemented ration prepared with unwashed Purina Assay Protein RP-100, containing approximately 100 μg zinc/g.

[4] Unicam SP-90 atomic absorption spectrophotometer with a detection limit of 0.02 ppm zinc.

[5] Purina Assay Protein RP-100, Ralston Purina Company, St. Louis, Mo.

[6] Composition of the basal salt mix (in grams): $CaCO_3$, 600; $Ca(H_2PO_4)_2 \cdot H_2O$, 220; K_2HPO_4, 650; NaCl, 336; $MgSO_4 \cdot 7H_2O$, 250; $FeSO_4 \cdot 7H_2O$, 50; $MnSO_4 \cdot H_2O$, 4.6; KI, 1.6; $CuSO_4 \cdot 5H_2O$, 0.6.

[7] A mixture of crystalline vitamins in glucose provided the following intake (μg/day): Ca pantothenate, 500; *p*-aminobenzoic acid and riboflavin each 100; thiamine HCl, pyridoxine and nicotinic acid, each 300; menadione, 250; folic acid, 6; biotin, 2.5; vitamin B_{12}, 0.3; choline chloride, 10 mg; inositol, 5 mg; ascorbic acid, 1 mg; α-tocopherol acetate, 1.2 IU; vitamin A palmitate, 150 IU; vitamin D_3, 15 IU.

gestation animals were anesthetized with chloroform and blood samples for plasma zinc analyses (10, 11) were withdrawn directly from the heart. At the same time, embryos were removed from each female by caesarian section and pooled for determination of the activity of thymidine kinase.

Assay of thymidine kinase. The pooled embryos were homogenized in approximately 12 vol of chilled 0.25 *N* Tris–HCl buffer pH 8.0, and an enzyme extract was prepared for use in the subsequent assays as described by Witschi (12). The activity of thymidine kinase was determined by measuring the amount of ^{3}H-thymidine monophosphate formed after incubating 0.1 ml of enzyme extract in 1.1 ml of a reaction mixture, pH 8.0, containing 132 μmoles Tris–HCl, 5.5 μmoles ATP, 6.6 μmoles 3-phosphoglyceric acid, 5.5 μmoles $MgCl_2$ and 600 pmoles (7.8 μCi) thymidine (^{3}H-methyl),[8] at 37° for 10 min (12, 13). ^{3}H-Thymidine monophosphate was separated onto 2.5 cm DEAE cellulose filter paper discs[9] and radioactivity was measured in a Nuclear Chicago liquid scintillation spectrometer.[10] The activity of thymidine kinase was expressed as pmoles thymidine phosphorylated/mg protein/hr.

All reagent solutions used in the course of the enzyme assays contained consistently less than 0.1 μg zinc/ml, but in certain experiments supplementary zinc [1.0 μg/ml (0.015 m*M*)–50 μg/ml (0.75 m*M*)] and other metal ions (0.015 and 0.75 m*M*) were added to the incubation mixture before addition of the ^{3}H-thymidine.

Protein assay. The concentration of protein in the fetal extracts was determined with Folin Ciocalteu's phenol reagent according to the method of Lowry *et al.* (14).

Results. The activity of thymidine kinase was reduced significantly in 12-day embryos taken from females exposed to a dietary zinc deficiency throughout the first 12 days of gestation when compared to both the *ad libitum* ($P < .05$) and restricted intake controls ($P < .01$) (see Table I). Although the activity of the enzyme had clearly fallen in animals receiving the zinc deficient diet for only 3 days, statistical significance was not attained with this group. Maternal plasma zinc levels fell by approximately 50% in both zinc depleted groups although in the animals receiving the deficient diet for only 3 days, two animals showed values rather higher than the rest (0.55 and 0.70 μg/ml).

The addition of zinc, as zinc chloride, to the assay medium had little effect on the activity of thymidine kinase in the zinc deficient extracts at levels between 0.015 and 0.075 m*M* (1.0–5.0 μg/g) but supplementation at 0.75 m*M* (50.0 μg zinc/ml) resulted in a 50% reduction in the rate of phosphorylation of ^{3}H-thymidine (see Table II). The activity of thymidine kinase from control fetuses was also not enhanced by the addition of zinc, and a similar inhibition was noted at higher concentrations (0.75 m*M*) as was observed with the deficient extracts.

In a further experiment (see Table III) the addition of calcium, cobalt, iron, magnesium, and manganese had little effect on the activity of thymidine kinase when added to the medium at concentrations that with zinc had been found to have either no effect (0.015 m*M*) or to be strongly inhibitory (0.75 m*M*). However, cadmium and copper reduced activity substantially at 0.015 m*M*, and at 0.75 m*M*, copper almost totally inhibited the activity of thymidine kinase.

Discussion. The present data on 12-day embryos confirm and extend previous reports (7, 8) concerning reduced activity of thymidine kinase in zinc deficient mammalian tissues.

The sharp fall in the activity of embryonic thymidine kinase after 12 days of maternal zinc restriction suggests that the impaired DNA synthesis and teratogenesis associated with zinc deficiency may arise primarily from reduced activity of this enzyme during organogenesis. Lowered thymidine kinase activity after as little as 3 days on the

[8] Thymidine [methyl^{3}H], 13 Ci/*M* obtained from Schwarz/Mann, Orangeburg, N. Y.

[9] Whatman DEAE Cellulose (DE81) filter paper circles (2.5 cm) obtained from Reeve Angel, 9 Bridewell Place, Clifton, N. J.

[10] Nuclear Chicago Mark I, 3 Channel, liquid scintillation system.

TABLE I. EFFECT OF MATERNAL ZINC DEFICIENCY ON ACTIVITY OF THYMIDINE KINASE IN 12-DAY RAT EMBRYOS.

Group	Number of litters	Maternal plasma zinc	Activity of thymidine kinase
		(μg/ml)	(pmoles thymidine monophosphate formed/mg protein/hr
Zinc-supplemented controls, *ad lib.*	6	0.86 ± 0.040[a]	729 ± 101[a]
Zinc-supplemented controls, restricted-intake	6	0.83 ± 0.021	950 ± 97.7
Zinc-deficient, *ad lib.*, Days 0–12	6	0.39 ± 0.061	356 ± 78.6[b,c]
Zinc-deficient, *ad lib.*, Days 9–12	6	0.44 ± 0.067	629 ± 125[d]

[a] Mean ± standard errors.
[b] $P < .05$ compared to *ad lib.* controls.
[c] $P < .01$ compared to restricted-intake controls.
[d] $P < .1$ compared to restricted-intake controls.

TABLE II. EFFECT OF SUPPLEMENTARY ZINC ON THE ACTIVITY OF THYMIDINE KINASE IN 12-DAY RAT FETUSES.

Zinc added (mM)	Percentage of original thymidine kinase activity	
	Zinc-deficient	Zinc-supplemented controls
0.015	99.03 ± 2.11[a]	91.57 ± 3.00[a]
0.075	97.60 ± 2.25	94.11 ± 3.09
0.75	46.84 ± 9.00	56.81 ± 7.92

[a] Means ± standard errors of determinations on three litters.

TABLE III. EFFECT OF SUPPLEMENTARY METAL IONS ON THE ACTIVITY OF THYMIDINE KINASE IN 12-DAY RAT FETUSES.

Metal ion added	Concentration (mM)	Percentage of original thymidine kinase activity	
		Zinc-deficient	Zinc-supplemented controls
Ca^{2+}	0.015	93.56±4.51[a]	96.93±0.69[a]
Ca^{2+}	0.75	99.72±2.86	95.40±5.69
Cd^{2+}	0.015	72.26±1.15	78.33±3.05
Cd^{2+}	0.75	50.10±2.49	35.00±2.41
Co^{2+}	0.015	94.01±3.76	94.63±1.86
Co^{2+}	0.75	90.76±1.78	86.03±3.33
Cu^{2+}	0.015	52.13±1.90	60.23±2.11
Cu^{2+}	0.75	9.66±2.92	6.66±0.44
Fe^{2+}	0.015	91.16±2.49	94.03±3.13
Fe^{2+}	0.75	83.83±8.52	79.96±4.97
Mg^{2+}	0.015	100.56±0.54	98.99±0.92
Mg^{2+}	0.75	97.46±0.92	102.70±2.45
Mn^{2+}	0.015	96.23±0.69	94.03±2.83
Mn^{2+}	0.75	98.20±1.39	82.03±3.25
Zn^{2+}	0.015	99.03±2.11	91.57±3.00
Zn^{2+}	0.75	46.84±9.00	56.80±7.92

[a] Means ± standard errors of determinations on three litters.

deficient diet, although not statistically proven, may account for the less severe teratogenesis observed by Hurley *et al.* (2) after a similar brief exposure.

Note that the thymidine kinase pathway for the production of thymidine nucleotides is not prominent in normal adult cells and it becomes important for DNA synthesis only in tissues undergoing rapid cell division (15). Since the effect of zinc deficiency on cell division has been observed to be most manifest in rapidly proliferating tissues, the relationship of zinc to thymidine kinase in the developing embryo might be expected to be of critical importance.

The failure of zinc, added at the time of assay, to restore the activity of thymidine kinase in zinc deficient extracts suggests that zinc is not associated with the enzyme as a readily dissociable cofactor. Reduction in activity of the enzyme therefore appears to involve either lack of incorporation of zinc into the enzyme at the time of synthesis, or the removal of zinc from the metalloenzyme in a more or less irreversible way. Since enzyme synthesis presumably occurred in a zinc deficient milieu, the former possibility seems more likely than the latter. Possibly

in the absence of zinc, synthesis of the apoprotein itself is diminished.

Duncan and Dreosti (16, 17) recently demonstrated that DNA synthesis was reduced by high levels of zinc in cultured rat lymphocytes (16) and transplanted rat tumors (17). The present inhibition of thymidine kinase *in vitro* by levels of zinc above 0.075 m*M* (5.0 μg/ml) suggests that the effect may arise from an insufficiency of thymidine monophosphate following diminished thymidine kinase activity of these tissues. The lack of specificity of zinc as an inhibitor of thymidine kinase is highlighted by the considerably greater reduction in enzyme activity following addition of cadmium or copper at concentrations of 0.75 m*M*. The effect of both metal ions is of interest as previous reports (18) concerning cadmium–zinc and copper–zinc interactions have indicated that copper and zinc are antagonistic, especially with regard to uptake and transmembranal transport.

The extreme sensitivity of thymidine kinase to levels of copper as low as 0.015 m*M* (0.96 μg/ml) suggests that the enzyme must be well insulated *in vivo* against upward fluctuations in ambient copper ion concentration, and raises the question whether the reduction in thymidine kinase activity in zinc deficient tissues might not arise from an increase in intracellular copper levels resulting from an effect of zinc deficiency on transmembranal copper transport. Alternatively, the effect may arise from a change in the relative copper:zinc ratio resulting from lowered intracellular zinc levels.

Recently, cadmium has been demonstrated to arrest growth in *E. gracilis* by interfering with the cell cycle and causing the production of multinucleate cells of high DNA content.[11] However, no effect was noted with respect to DNA syntheses as has been reported in the present study. Perhaps cadmium did not reach the site of thymidine kinase activity in the intact cell system.

The experiments reported here suggest that the teratogenic effects of zinc deficiency in rats arise from impaired activity of fetal thymidine kinase during embryonic morphogenesis. Activity of the enzyme was not restored by the *in vitro* addition of zinc and the severe inhibition of thymidine kinase by copper points to the possibility of changes in intracellular copper concentration contributing to the inhibition of the enzyme.

Summary. The activity of thymidine kinase in 12-day fetuses taken from females exposed to a dietary zinc deficiency during pregnancy was significantly lower than in *ad libitum* ($P < .05$) and restricted-intake ($P < .01$) controls. Activity of the enzyme was not restored by *in vitro* addition of zinc at levels up to 0.075 m*M* but severe inhibition (approximately 50%) occurred at 0.75 m*M*. Enzyme activity was also severely reduced (approximately 44%) by 0.017 m*M* (0.96 μg/ml) of copper which raises the possibility that the reduction in thymidine kinase accompanying zinc deficiency may arise, at least in part, from an absolute or relative change in the intracellular level of copper.

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