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Thymidine Kinase and DNA Polymerase Activity in Normal and Zinc Deficient Developing Rat Embryos (40279)

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In rapidly dividing cells, DNA synthesis is inhibited by zinc deficiency (1–5). It has been suggested that the severe teratogenesis resulting from maternal dietary zinc deficiency in rats may arise as a consequence of impaired DNA synthesis during fetal organogenesis (2, 3). Studies on regenerating rat liver (6), tumor tissue (7), and rat connective tissue (8) have indicated that the decrease in DNA synthesis as a result of zinc deficiency may be linked to an association of zinc with one or more of the zinc dependent enzymes involved in DNA synthesis. The activity of two regulatory enzymes in DNA synthesis, thymidine kinase and DNA polymerase (9), was found to be reduced in zinc deficient rats (7, 8). Thymidine kinase is of particular importance since zinc deficiency produced a significant reduction in activity of this enzyme in regenerating rat liver within 10 hr after partial hepatectomy, whereas DNA polymerase activity, DNA synthesis, and protein synthesis were not affected until some hours later (10). Recently, Dreosti and Hurley (11) found the activity of thymidine kinase to be significantly lower in embryos taken at 12 days of gestation from zinc deficient dams than in those from controls.

In the present study, the effect of zinc deficiency on the activity of DNA polymerase was investigated in 12-day embryos. In addition, since a previous report suggested that early embryos are relatively less sensitive to zinc deficiency than are older embryos (12), the activity of both thymidine kinase and DNA polymerase was measured in 9, 10, and 11 day embryos from zinc deficient and control rats to determine whether zinc was acting at similar sites at early stages of gestation. Further studies were also undertaken to test the effect of in vitro supplementation with zinc and other divalent metal ions on the activity of DNA polymerase. Similar data relating to thymidine kinase have been reported previously (11).

Materials and methods. Materials. (Methyl-^{3}H) thymidine (spec. act. 2 Ci/m*M*) and (methyl-^{3}H) thymidine 5′-triphosphate (spec. act. 15 Ci/m*M*) were purchased from Amersham/Searle Corporation, Arlington Heights, Illinois. All other chemicals were purchased from Sigma Chemical Company, St Louis, MO. Whatman DEAE cellulose (DE 23) filter paper circles were obtained from Reeve Angel, 9 Bridewell Place, Clifton, NJ.

Animals and diets. Virgin female Sprague-Dawley rats weighing 210 ± 10 g were bred overnight with stock fed 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. The animals were fed a zinc deficient diet *ad libitum*, or a control diet *ad libitum*, or a control diet in amounts limited to the mean daily food intake of the deficient group (referred to as "restricted intake").

The zinc deficient diet contained less than 0.5 ppm zinc as measured by atomic absorption spectroscopy. The control diet was the same purified diet as the zinc deficient diet except that it was supplemented with zinc as zinc carbonate to a level of 100 μg/g. The composition of the diet has been described previously (13). In addition, all animals received vitamins in glucose three times per week.

Collection of samples. On day 9, 10, 11, or 12 of gestation, the animals were killed and embryos were removed by caesarean section. In order to obtain sufficient tissue for the enzyme assay it was necessary to pool litters of embryos. Five litters were pooled for each 9-day sample, three litters for each 10-day sample, two litters for each 11-day sample, one litter for each 12-day sample.

Enzyme assays. Pooled embryos were homogenized in 12 vol of chilled 0.25 *N* Tris–HCl buffer, pH 8.0, and an enzyme solution was prepared for use in the subsequent assays as described by Witschi (14).

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Thymidine kinase was assayed by a modified procedure described by Witschi (14). The reaction mixture contained in a final volume of 0.5 ml, 0.25 *N* Tris–HCl buffer (pH 8.0), 5.5 μM ATP, 6.6 μM 3-phosphoglyceric acid, 5.5 μM $MgCl_2$ and 2.5 μM (5.0 μCi) (methyl-^{3}H) thymidine and 0.1 ml of the enzyme extract. The reaction mixture was incubated at 37° for 15 min and the reaction was stopped by immersing the assay tubes in boiling water for 1 min. After cooling and centrifugation at 1000*g* for 10 min, 50 μl aliquots of the protein-free supernatants were spotted onto DEAE cellulose filter paper discs and the papers were washed in 1.0 m*M* ammonium formate, water, and 95% ethanol. Radioactivity on the dried paper discs was measured in a Nuclear Chicago Mark I liquid scintillation spectrophotometer.

DNA polymerase was determined by a modified procedure described by Witschi (14) and Lehman *et al.* (15). The reaction mixture contained in a final volume of 0.5 ml, 0.25 *N* Tris–HCl buffer (pH 8.0), 0.05 μM d-ATP, 0.05 μM d-CTP, 0.05 μM d-GTP, 1.5 μM $MgCl_2$, 1.5 μM KCl, 0.05 μM 2-mercaptoethanol, 50 μg heat denatured DNA (70° for 15 min), 0.05 μM (5 μCi) dTTP and 0.1 ml of the enzyme extract. After incubation at 37° for 1 hr, the reaction was stopped by the addition of 0.1 ml cold 1.0 *M* $HClO_4$. The precipitate was washed twice with 0.5 *M* $HClO_4$, dissolved in 0.3 *M* KOH (3 ml), incubated for 60 min at 37°, reprecipitated with cold 0.5 *M* $HClO_4$, and washed once more. The pellet was dissolved in 1 *M* NaOH (2 ml) and 0.5 ml aliquots were withdrawn for radioactivity determinations.

Metal ion supplementation. In certain DNA polymerase assays, supplementary zinc and other metal ions (0.01–0.2 mM) were added to the incubation mixture before addition of ^{3}H-dTTP. All metal salts used were spectrophotometrically pure and, except for the zinc salt, contained less than 0.05 μg zinc/g.

Protein assay. The concentration of protein in the fetal homogenates was determined by the method of Lowry *et al.* (16).

Statistical analysis. Mean ± SEM are reported. The statistical significance of differences between means was tested by Student's "t" test.

Results. The activity of thymidine kinase was significantly lower in 9, 10, 11, and 12 days embryos taken from females fed a zinc deficient diet than in embryos from either ad libitum fed ($P < 0.05$) or restricted intake ($P < 0.05$) controls (Table I). However, the percentage decrease in activity in the zinc deficient animals when compared with restricted intake controls was not as great in early embryos as in the 12-day embryos. In addition, the activity of thymidine kinase increased with increasing age of embryos in all three dietary groups (Table I). The percentage increase in activity was greatest at early stages of gestation. Activity in the 9-day groups was only twice that of background values.

DNA polymerase activity was also significantly lower in 9, 10, 11, and 12 day embryos from dams fed the zinc deficient diet than in embryos from either the *ad libitum* fed ($P <$

TABLE I. EFFECT OF ZINC DEFICIENCY AND DAY OF GESTATION ON ACTIVITY OF THYMIDINE KINASE IN RAT EMBRYOS.[a]

	Groups						
	Control *ad libitum*		Control restricted intake		Zinc deficient		
Day of gestation	Activity	Daily increase in activity (%)	Activity	Daily increase in activity (%)	Activity	Daily increase in activity (%)	% of control restricted intake
9	79 ± 9**		81 ± 14**		64 ± 6*,**		79
10	323 ± 66**	309	351 ± 52**	333	196 ± 32*,**	206	56
11	665 ± 95	105	659 ± 101**	88	372 ± 76*	90	56
12***	729 ± 101	10	950 ± 48	44	356 ± 79*	—	37

[a] Thymidine kinase activity expressed as p*M* ^{3}H-thymidine incorporated/mg protein/hr.

* $P < 0.05$ compared to *ad libitum* and restricted intake controls.

** $P < 0.05$ compared to activity in 1-day older embryos in the same group.

*** Data from Dreosti, I. E., and Hurley, L. S., Proc. Soc. Exp. Biol. Med. **150,** 161 (1975).

0.01) or restricted intake ($P < 0.01$) controls (Table II). The percentage decrease in the zinc deficient groups when compared with restricted intake controls was similar at all 4 days of gestation. DNA polymerase activity also increased with increasing age of embryos in all three dietary groups, but the percentage daily increase was not as great as that found with thymidine kinase (Table II). Even at 9 days of gestation, embryos had appreciable levels of DNA polymerase activity.

Addition of zinc, as zinc chloride, to the assay medium (at levels between 0.01 m*M* and 0.05 m*M*) had little effect on the activity of DNA polymerase in extracts from zinc deficient and control embryos at 12 days of gestation (Table III). However, supplementation of these extracts with a higher level, 0.2 m*M* zinc, resulted in a statistically significant depression of activity in extracts from both zinc deficient and control embryos (19% and 21%, respectively).

In a further experiment (Table IV), addition of Cu^{2+}, Cd^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, and Fe^{2+} had no effect on the activity of DNA polymerase when added to the medium at concentrations of 0.01 m*M* or 0.2 m*M*.

Discussion. The low activity of thymidine kinase and DNA polymerase in embryos from zinc deficient dams confirms previous reports of reduced activity of these enzymes in zinc deficient mammalian tissues (6–8). It further suggests that impaired DNA synthesis and teratogenesis associated with zinc deficiency may be related to reduced activity of these enzymes during organogenesis.

The thymidine kinase salvage pathway is important for DNA synthesis only in rapidly dividing cells, not in normal adult cells where the *de novo* pathway of DNA synthesis is predominant (9, 17). Therefore, the thymidine kinase pathway may be of critical importance in the developing embryo. Since the effect of zinc deficiency on cell division is most manifest in rapidly proliferating tissues, it is reasonable to suppose that thymidine kinase may be involved. In contrast, while the activity of certain DNA polymerase enzymes is enhanced in rapidly dividing cells, these enzymes, unlike thymidine kinase, are also important in DNA synthesis in normal resting cells (18, 19).

Further support for the idea that thymidine kinase, and possibly DNA polymerase, are possibly primary sites of action of zinc in embryonic tissue is provided by the finding of decreased activity of both enzymes with decreasing age of embryos. Hurley *et al.* (12) have found a low incidence of congenital

TABLE III. EFFECT OF SUPPLEMENTARY ZINC ON THE ACTIVITY OF DNA POLYMERASE IN 12-DAY RAT EMBRYOS.[a]

Zinc added (m*M*)	Percent of original DNA polymerase activity	
	Zinc supplemented control	Zinc deficient
—	100 (±3.5)	100 (±3.2)
0.01	106 (±2.7)	113 (±4.5)
0.05	114 (±6.3)	121 (±7.3)
0.2	79 (±3.7)*	81 (±3.1)*

[a] DNA polymerase activity expressed as n*M* ^{3}H-TTP incorporated/mg protein/hr.

* $P < 0.05$ compared to extracts with no zinc added.

TABLE II. EFFECT ON ZINC DEFICIENCY AND DAY OF GESTATION ON ACTIVITY OF DNA POLYMERASE IN RAT EMBRYOS.[a]

	Groups						
	Control *ad libitum*		Control restricted intake		Zinc deficient		
Day of gestation	Activity	Daily increase in activity (%)	Activity	Daily increase in activity (%)	Activity	Daily increase in activity (%)	% of control restricted intake
9	2.32 ± 0.12**		2.19 ± 0.24**		1.44 ± 0.30*,**		67
10	2.68 ± 0.25**	16	2.51 ± 0.22	15	1.62 ± 0.25*,**	13	66
11	2.96 ± 0.28	10	2.62 ± 0.42	4	1.88 ± 0.25*	16	72
12	3.06 ± 0.35	3	2.59 ± 0.38		1.97 ± 0.26*	5	76

[a] DNA polymerase activity expressed as n*M* ^{3}H-TTP incorporated/mg protein/hr.

* $P < 0.01$ compared to *ad libitum* and restricted intake controls.

** $P < 0.05$ compared to activity in 1-day older embryos in the same group.

TABLE IV. EFFECT OF SUPPLEMENTARY METAL IONS ON THE ACTIVITY OF DNA POLYMERASE IN 12-DAY EMBRYOS FROM ZINC DEFICIENT DAMS.[a]

Metal ion added	Concentration (m*M*)	Percent of original DNA polymerase activity
—	—	100 (±3.5)
Cu^{2+}	0.01	92 (±3.6)
	0.2	85 (±6.8)
Cd^{2+}	0.01	95 (±4.9)
	0.2	90 (±2.8)
Mn^{2+}	0.01	98 (±3.7)
	0.2	98 (±5.7)
Mg^{2+}	0.01	100 (±8.0)
	0.2	99 (±4.7)
Co^{2+}	0.01	100 (±5.8)
	0.2	96 (±3.9)
Fe^{2+}	0.01	97 (±9.1)
	0.2	93 (±4.0)

[a] DNA polymerase activity expressed as n*M* ^{3}H-TTP incorporated/mg protein/hr.

abnormalities in rats fed a zinc deficient diet from days 0 to 8 of pregnancy. The incidence of malformations increased when the animals were fed a zinc deficient diet for longer periods during gestation or for the same length of time but at a later stage of gestation. The very low activity of thymidine kinase in rat embryos at 9 days of gestation, together with the relatively smaller decrease in enzyme activity in 9-day embryos than in 12-day embryos from zinc deficient animals, may therefore make the early embryo relatively less sensitive to zinc deficiency than are later embryonic stages.

The failure of zinc added at the time of assay to restore the activity of DNA polymerase in zinc deficient enzyme extracts confirms earlier observations with extracts from regenerating rat liver (10) and suggests that zinc may not be associated with the enzyme as a readily dissociable cofactor. This finding is similar to observations with thymidine kinase and may be explained by a lack of incorporation of zinc into the enzyme at the time of synthesis. The possibility of reduced synthesis of the enzyme as a result of general reduced protein synthesis in the zinc deficient animals is unlikely since it has been shown in regenerating rat liver that protein synthesis was not affected by zinc deficiency until 10–20 hr after a change in DNA polymerase activity was noted (10).

The inhibitory effect of a high level of zinc (0.2 m*M*) on DNA polymerase activity *in vitro* was similar to that reported by Dreosti and Hurley (11) for thymidine kinase. Such inhibition of activity of these two enzymes may account for the reduced DNA synthesis produced by high levels of zinc in cultured rat lymphocytes (20) and transplanted rat tumors (21).

Unlike thymidine kinase, which was relatively sensitive to Cd^{2+} and Cu^{2+}, addition of various metal ions at both low (0.01 m*M*) and high (0.2 m*M*) concentrations had little effect on the activity of DNA polymerase in vitro. This observation supports the data of Springgate *et al.* (22) using a zinc free apoenzyme and suggests that DNA polymerase is specifically zinc dependent.

In conclusion, the findings reported here indicate that the teratogenic effects of zinc deficiency in rats may arise from impaired activity of fetal thymidine kinase and DNA polymerase after day 8 of gestation and that the primary effect may be on the regulatory enzyme, thymidine kinase. The *in vitro* addition of metal ions to zinc deficient enzyme extracts suggests that zinc may not be associated with DNA polymerase as a readily dissociable cofactor and that DNA polymerase is specifically zinc dependent.

Summary. Thymidine kinase and DNA polymerase activities were significantly ($P < 0.05$ and $P < 0.01$, respectively) lower in 9, 10, 11, and 12-day embryos taken from dams fed a zinc deficient diet than in those from *ad libitum* fed and restricted intake controls. An additional finding was that of increased activity of both thymidine kinase and DNA polymerase with increasing age of embryos. As previously found with thymidine kinase, addition of zinc and other divalent metal ions *in vitro* had little effect on restoration of DNA polymerase activity from zinc deficient extracts when added at concentrations of 0.01 and 0.05 m*M*. When added at a level of 0.2 m*M*, zinc, but not other metal ions, had an inhibitory effect on DNA polymerase activity. These findings support the hypothesis that the teratogenic effects of zinc deficiency are associated with the enzymes involved in DNA synthesis.

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