Our method should be useful selecting variants lacking membrane-associated Ig or in characterizing the structure of membrane-associated Ig subunits.

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Teratogenic Effects of a Chelating Agent and Their Prevention by Zinc

Abstract. *Ingestion of a chelating agent (ethylenediaminetetraacetic acid) by female rats during pregnancy impaired reproduction and resulted in congenitally malformed young. When ethylenediaminetetraacetic acid was fed from days 6 to 21 of gestation, all of the full-term young had gross congenital malformations. These effects were prevented by simultaneous supplementation with 1000 parts per million of dietary zinc.*

Increased use of metal-binding substances in medicine (*1*) has stimulated interest in the potential toxic effects of ethylenediaminetetraacetic acid (EDTA) and related chelating compounds. Recent concern over the proposed use of nitrilotriacetic acid in detergents exemplifies heightened awareness of possible health hazards in widespread use of chelates.

Early evidence of chelate toxicity appeared in 1956, shortly after synthetic chelating compounds became available. Injection of EDTA into pregnant rats resulted in congenital malformations in the young (*2*). Since it was known that this chelating agent binds calcium, the investigators suggested that impairment of fetal development might be due to maternal hypocalcemia (*2*). However, EDTA also complexes zinc (as well as other divalent cations), and the observed teratogenic effects of EDTA may have been due to an induced deficiency of zinc rather than of calcium. Indeed,

even a short-term deficiency of dietary zinc during pregnancy has been shown to result in gross congenital malformations in rats (*3*). In contrast, calcium deficiency is not known to be teratogenic even in parathyroidectomized rats (*4*).

In Japanese quail (*Coturnix coturnix japonica*), the addition of small amounts of EDTA improved the hatchability of eggs (*5*, *6*), but higher levels of EDTA reduced hatchability and resulted in some malformations (*5*). In the present investigation, purified diets containing 2 and 3 percent EDTA salts were fed to pregnant rats to determine the effect of dietary EDTA on development of the embryo. The influence of zinc in preventing the effects of EDTA was also tested.

The control ration had the following composition (in percent): soybean protein (*7*), 30.0; sucrose, 57.3; corn oil, 8.0; salt mix (*8*), 4.0; and DL-methionine, 0.7. This ration contained 100 parts per million (ppm) of zinc (provided as zinc carbonate in the salt mix). Crystalline vitamins were given separately (*9*). The EDTA-containing diets were prepared by adding either 2 or 3 g of the disodium salt of EDTA (Na_2EDTA) to 100 g of the control ration (designated as 2 or 3 percent EDTA diets, respectively). Some of the 3 percent EDTA diet was supplemented with additional zinc carbonate, which provided a total content in the diet of 1000 ppm of zinc. The animals had free access to deionized water and food.

Female Sprague-Dawley rats weighing 210 ± 10 g were purchased from a commercial source and were fed the purified control ration for at least 5 days before breeding. Estrous cycles were determined by daily vaginal smears, and the animals were mated overnight with normal stock-fed (*10*)

Table 1. The effect of dietary EDTA supplementation on reproduction in rats. The period of Na_2EDTA ingestion is given as the days of gestation.

| Dietary zinc (ppm) | Dietary Na_2EDTA* | | Rats (No.) | | | Implantation sites | | | Living young at term | | | |
	Percent	Period of ingestion	Mated	With implantation sites	With living young at term	Total No.	Dead or resorbed fetuses (%)	Affected† (%)	Total No.	Mean No. per litter‡	Mean weight of young (g)	Malformed (%)
100	0	0 (controls)	11	11	11	132	6	6	124	11.4	5.3	0
	2	0 to 21	5	5	5	61	5	11	58	11.6	4.6	7
	3	0 to 21	8	0	0	0			0			
		6 to 14	11	10	8	103	40	97	70	7.0	3.7	87
		6 to 21	16	16	11	182	54	100	183	5.5	1.8	100
1000	3	6 to 21	8	7	7	88	8	8	81	11.6	5.0	0

* Percentage is expressed as grams of Na_2EDTA added to 100 g of diet. † Percent of implantation sites having dead, resorbed, or malformed fetuses. ‡ Mean number of rats with implantation sites.
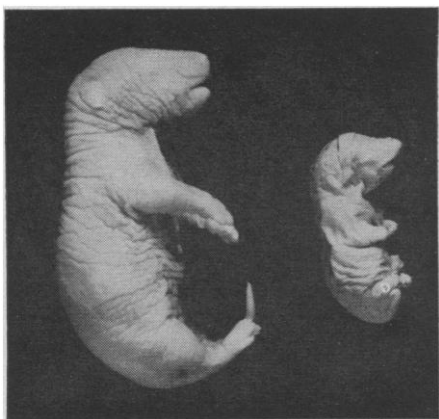
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Fig. 1. An example of congenital malformations in the full-term fetus from a female rat given 3 percent dietary EDTA from days 6 to 21 of gestation (right) as compared with normal control (left). Note small size, domed head, small lower jaw (micrognathia), clubbed legs, fused or missing digits (syndactyly), and stubby, curly tail. (Razor cuts on the head were made for examination of eye and brain.)

males. Matings were confirmed by the presence of sperm in the vaginal smear. The day of finding sperm was considered day 0 of gestation. Female rats were housed individually in stainless steel cages and racks. Details of the procedures used have been described previously (11).

In one experiment, female rats were fed either the 2 or the 3 percent EDTA diet from day 0 of gestation to term (day 21). Control rats were fed the control diet without added Na₂EDTA. In another experiment, rats were fed the 3 percent EDTA diet either from days 6 to 21 or from days 6 to 14 of pregnancy, and then given the control diet until term. One group of rats was fed the 3 percent EDTA ration with 1000 ppm of zinc from days 6 to 21. On the last day of gestation (day 21), fetuses were removed by cesarean section, fixed in Bouin's solution for 48 hours, and stored in 70 percent ethanol. Gross examination for external congenital abnormalities was made with the aid of a dissecting microscope. Eye and brain were examined in razor cut sections of the head. Fetuses were not examined for other internal abnormalities. The number of implantation sites was determined by counting the number of metrial nodes in the uterus.

Data from the two experiments have been combined in Table 1. All females fed diets containing EDTA salts had from moderate to severe diarrhea. When females were fed the 2 percent

EDTA diet throughout pregnancy, reproduction was impaired only slightly. All rats had living young at term and litter size was normal, although the young were slightly smaller than controls. However, 7 percent of the full-term young were malformed, while none of the control fetuses showed gross congenital malformations. When females were fed the 3 percent EDTA diet throughout pregnancy, reproduction was so severely disturbed that none of the mated females had grossly visible implantation sites at term.

When the 3 percent EDTA diet was fed from days 6 to 14 of gestation or from day 6 of gestation to term, almost all of the mated females had implantation sites, but nearly half of these sites had dead or resorbed fetuses. Females fed the 3 percent EDTA diet from days 6 to 21 had less than half the normal number of young per litter, and full-term young had a mean body weight of only 1.8 g as compared with 5.3 g in controls; furthermore, 100 percent of the young were grossly malformed (Fig. 1). In contrast, females given 1000 ppm of dietary zinc along with the same 3 percent EDTA for the same period of time during gestation had essentially normal reproduction and none of the young were malformed.

The incidences of various types of malformations observed when 3 percent EDTA was fed from days 6 to 21 of gestation are shown in Table 2. When this regimen contained 100 ppm of dietary zinc, 44 percent of the young had severe brain malformations, 57 percent had cleft palate or malformed digits, and nearly all had clubbed legs or malformed tails. None of these anomalies occurred in young from females fed the 3 percent EDTA diet with 1000 ppm of zinc.

These results show that Na₂EDTA ingested during pregnancy was teratogenic. Supplementation with zinc (1000 ppm) prevented the detrimental effects of EDTA. The effects of 3 percent dietary EDTA from days 6 to 21 of gestation were similar to those reported earlier in females given diets severely deficient in zinc throughout pregnancy (impaired reproduction and incidence of congenital malformations) (3). These findings suggest that the severe effects of EDTA may be due to an induced deficiency of zinc.

In rats, tissue stores of zinc cannot be mobilized in amounts adequate to maintain normal plasma levels of zinc

Table 2. Incidence of congenital malformations in rats receiving 3 percent dietary EDTA and two levels of dietary zinc.

	Dietary zinc (ppm)	
	100	1000
Number of litters	11	7
Number of fetuses	83	81
Malformations (percent of fetuses)		
Cleft lip	1.2	0
Cleft palate	57	0
Brain*	44	0
Micro- or anophthalmia	18	0
Micro- or agnathia	63	0
Clubbed legs	92	0
Fused or missing digits	57	0
Curly, short, or missing tail	98	0

* Hydrocephalus, anencephalus, hydranencephalus, or exencephalus.

or to support normal fetal development when dietary zinc is lacking (12). Cells undergoing rapid growth and development appear to be particularly sensitive to a deficiency of zinc (13). In humans, therapeutic injections of EDTA salts have resulted in a sixfold increase of urinary zinc (14). It would appear that nutritional status with respect to zinc should be considered whenever EDTA or related chelates are administered and especially during pregnancy.

The present study suggests but does not prove that the congenital anomalies caused by EDTA are due specifically to zinc deficiency. It is also possible that the preventive effect of additional zinc may be due to the binding of EDTA in the intestine so that the chelating agent is unable to complex some other essential element.

The concepts of dietary balance and interrelationships among nutrients are now well accepted. More recently, it has become evident that nutritional requirements can also be modified by substances in the environment that are not themselves nutrients. Because of the ubiquitous presence of zinc in plants and animals, deficiencies of this essential trace element have been considered rare in man. However, increases in environmental levels of metal-binding substances (such as EDTA) or zinc antagonists [such as cadmium (15)] may induce zinc deficiency and interfere with the fundamental processes in which the trace element plays an essential role.

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9. A mixture of crystalline vitamins in glucose was given three times each week in amounts to provide the following intake (in micrograms per day): Ca pantothenate, 500; *p*-aminobenzoic acid and riboflavin, each 100;
thiamine·HCl, pyridoxine·HCl, and nicotinic acid, each 300; menadione, 250; folic acid, 6; biotin, 2.5; vitamin B_{12}, 0.3; and choline chloride, 10 mg; inositol, 5 mg; ascorbic acid, 1 mg; α-tocopheryl acetate, 1.2 international units (I.U.); vitamin A palmitate, 150 I.U., and vitamin D_3, 15 I.U. During pregnancy the vitamin supplement was doubled.
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Angiotensin-Forming Enzyme in Brain Tissue

Abstract. A renin-like enzyme is present in brain tissue and is independent of kidney and plasma renin. In the presence of homologous substrate it forms angiotensin. Administration of aldosterone significantly decreases this angiotensin-forming enzyme activity, while administration of progesterone markedly enhances it.

Angiotensin is the most potent compound known to stimulate fluid intake through its effect upon certain brain regions (*1*), and angiotensin-sensitive brain receptors respond to changes in plasma Na^+ to control body fluid and electrolyte balance (*2*). Release of antidiuretic hormone, catecholamines, and acetylcholine from nerve cells can be increased by angiotensin (*3*). To date, however, local formation of angiotensin in brain has not been demonstrated. We report here our findings of such a kidney-independent angiotensin-forming enzyme in brain tissue.

Angiotensin formation by tissues other than the kidney is already known. Recently we have shown that tissue renin activity in the mesenteric arterioles of nephrectomized dogs could be markedly increased following severe bleeding (*4*). Tissue renin activity has been found to persist and even increase after nephrectomy (*5*), and could be due to local tissue synthesis. In vitro production of renin in uterine muscle has also been described (*6*). Recent studies in our laboratory show that renin does not disappear in brain and arterial tissues 12 days after bilateral nephrectomy in dogs kept alive by peritoneal dialysis.

Adult male mongrel dogs were used for all experiments. Tissue renin activity and plasma renin activity were measured by the micromethod of Boucher
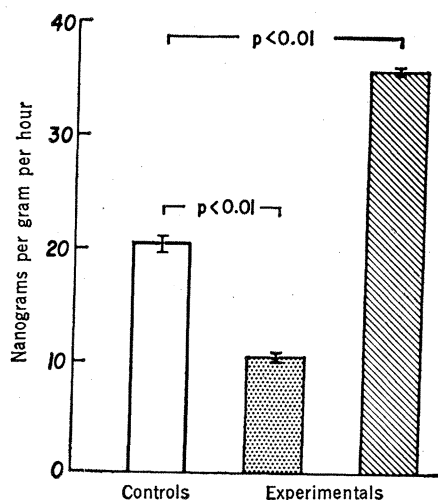

Fig. 1. Changes in angiotensin-forming enzyme in brain tissue (left caudate nucleus), expressed in nanograms of angiotensin formed per gram wet weight of tissue per 1 hour of incubation at 37°C. Stippled column: values after 8 days of administration of aldosterone (2 mg/day). Hatched column: values after 4 weeks of administration of progesterone (25 mg/1 day).

et al. (*7*), in which there is no interference by dopamine, norepinephrine, epinephrine, serotonin, acetylcholine, γ-aminobutyric acid, or histamine during the incubation. The angiotensin-forming enzyme in brain (caudate nucleus, frontal cortex, thalamus, hypothalamus, and brainstem) and arteries have the same characteristics. (i) Like renal renin, it is destroyed by heat, is nondialyzable, acts on homologous substrate to form a pressor substance with or without the presence of plasma, and is precipitated by ammonium sulfate. (ii) Without substrate, there is no formation of pressor material. (iii) The amount of pressor material formed is linear with respect to the amount of substrate and the time of incubation. (iv) It is stimulated by sodium depletion [from 2.49 ng g^{-1} $hr^{-1} \pm 0.3$ (S.E.) to 7.72 ng g^{-1} $hr^{-1} \pm 0.64$ (S.E.) in mesenteric artery branches]. (v) The enzyme activity can be inhibited by anti-hog renin in dog's plasma (33.4 percent inhibition for brain enzyme and 77.5 percent inhibition for kidney renin of the dog has been obtained) (*8*). (vi) The pressor material formed by renal renin and by brain and arterial tissue renin is dialyzable, heat-stable, inactivated by trypsin, and adsorbed on Dowex 50 W-X2 (NH_4^+) and Dowex 50 W-X8 (H^+). (vii) The pressor effect in the rat is identical to that of synthetic standard angiotensin II (Hypertensin, Ciba). The *p*H optimum for brain and arterial tissue renin is at *p*H 5.0, while the optimum for renal cortex renin of the dog is between *p*H 5.5 and 6.5 under the incubation conditions.

The effectiveness of the blood-brain barrier for renin (molecular weight about 45,000) was checked in 15 dogs with high levels of plasma renin under different conditions, such as sodium depletion, dehydration, and homologous renin infusion. In none of these experiments could renin be detected in the cerebrospinal fluid. This is in agreement with the observation that the blood-brain barrier excludes proteins as large as renin (*9*).

The enzyme activity in brain caudate nucleus tissue of control dogs is nearly ten times higher (20.72 ng g^{-1} hr^{-1}) than in mesenteric artery branches (2.49 ng g^{-1} hr^{-1}), whereas the control values of renal cortex renin is 23.5 μg g^{-1} hr^{-1}.

Subcellular fractionation by differential centrifugation and density gradient centrifugation (*10*) showed the tissue renin of the caudate nucleus to