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Effects of Lead Exposure on Skeletal Development in Rats

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The effects of lead on growth in female rats and on growth and skeletal development in their offspring were investigated. No alteration in growth rate, compared to the growth rate in pair-fed controls, was observed in 48 weanling females continuously exposed to 250 or 1000 ppm lead in drinking water and fed a replete diet. After 49 days of exposure, all rats (24 pair-fed controls, 12 exposed to 250 ppm lead, and 12 exposed to 1000 ppm lead) were mated with control males. At parturition, six lactating dams each from the 250 and 1000 ppm lead groups were removed from lead exposure and given control drinking water, and six lactating dams each from the control group were given either 250 or 1000 ppm lead in drinking water. Exposure conditions for the remaining dams in the control, 250, and 1000 ppm groups were not changed. Maternal blood lead in the continuously lead-exposed groups was higher at the end of lactation than prior to mating. Lead exposure prior to parturition caused greater maternal tibial lead accumulation than lead exposure after parturition. In contrast, lead exposure prior to parturition had a lesser impact on offspring tibial lead accumulation than lead exposure after parturition. Decreases in tibial calcium and phosphorus were observed in dams exposed continuously to 250 or 1000 ppm lead; however, there was no apparent effect of lead on maternal growth-plate morphology or on growth-plate width. Offspring body weight was depressed relative to controls during suckling (Day 11) and after weaning (Day 24) in high-dose and continuously lead-exposed groups. Continuous lead exposure caused a greater decrease in offspring body weight than lead exposure only prior to or after parturition. Decreased tail length growth suggested possible effects of lead on tail vertebral bone growth. While tibial calcium and phosphorus levels were not changed in the weanlings, increased weanling growth-plate width, with disruption of chondrocyte organization, and wider metaphyseal trabeculae were observed. Although the mechanisms of these effects are not known, the results suggest that local lead-related effects on growth-plate chondrogenesis and metaphyseal mineralization may be involved.

The skeleton is increasingly recognized as an important target for lead toxicity (Nordberg *et al.*, 1991; Silbergeld, 1991). Analysis of the National Health and Nutrition II Survey has demonstrated a negative relationship of blood lead in the range of 8 to 35 μg/dl with height, chest circumference, and body weight in children (Schwartz *et al.*, 1986). A pilot retrospective study of growth in children has recently shown that blood lead levels over 30 μg/dl and persistently high erythrocytic protoporphyrin (EP) levels were associated with reduced height and weight gain after age 24 months (Angle and Kunzelman, 1989). In addition, maternal lead exposure during pregnancy has been associated with increased cranial vault densities (abnormal mineralization of the skull) and delayed body development at birth (Pearl and Boxt, 1980). Lead is associated with reduced growth during early childhood (Mooty *et al.*, 1975), and lead exposure *in utero* may enhance lead-related growth retardation when continued postnatally (Shukla *et al.*, 1989).

Lead administered intravenously to the dam causes skeletal malformations in fetal mice and rats (McClain and Becker, 1975; Kennedy *et al.*, 1975). Increased trabecular width and osteoclast inclusion bodies were observed in femurs from pigs given lead in the diet (Hsu *et al.*, 1973). Bone formation was decreased in dogs exposed to dietary lead for 7 months; this effect was accompanied by decreased osteoblast activity for up to 6 months after cessation of exposure (Anderson and Danylchuk, 1980). Evidence of lead-related skeletal growth retardation has been reported, as indicated by nonclosure of vertebral growth plates in dogs fed lead (Stowe *et al.*, 1973). The "lead line" can be observed by radiography of the epiphyseal growth plate in long bones of lead-exposed humans (Park *et al.*, 1933), and a recent study has shown that lead can bind to growth-plate cartilage matrix sites normally associated with calcium and phosphorus (Arsenault and Hunziker, 1988).

In utero lead exposure combined with exposure after parturition has caused decreased body weight gain in rat offspring (Grant *et al.*, 1980). Results from a study with rats fed *ad libitum* suggest that lead-related effects on food intake should be considered in studies of lead effects on

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growth (Hamilton *et al.*, 1994). However, mechanisms of lead-related effects on growth and skeletal development are not well understood. Lead exposure has been associated with decreased growth rate in pair-fed weanling rats exposed to 50 or 250 ppm lead in drinking water (Grant *et al.*, 1980). Reduction in growth (body weight and tail length) associated with up to 1000 ppm lead in drinking water has also been observed in weanling rats fed *ad libitum* (Hamilton *et al.*, 1994). This study provides an assessment of the effect of lead on growth of pair-fed female rats exposed to 250 or 1000 ppm lead in drinking water and on skeletal development in their offspring.

METHODS

Prior to parturition. Forty-eight female and 15 male Sprague–Dawley cesarean-delivered (CD) rats (Charles River, Portage, MI) were obtained at age 21 days and singly housed in stainless steel wire cages. The housing cycle was 12 hr light and 12 hr dark under controlled temperature (22–25°C). After 4 days to allow acclimatization, the rats were assigned randomly to study groups. Twelve males and 24 females were assigned to the control group, and 12 females each were assigned to the 250 and 1000 ppm lead exposure groups (Fig. 1). Initial (Day 0) weights were matched as closely as possible within pair-fed control and lead-exposed female pairs. Twelve females in the control group were pair-fed daily to weight-matched 250 ppm group females, and the remaining 12 females in the control group were pair-fed daily to weight-matched 1000 ppm group females. Daily food intake correction was made to account for exposure-group food wastage. All males were fed *ad libitum* for later use in mating. Prior-to-parturition study groups are shown in Fig. 1.

Lead acetate trihydrate was dissolved in deionized water to give drinking water concentrations of 250 or 1000 ppm lead. Acetic acid (0.00125%) was added to aid in dissolution; the control group drinking water was 0.00125% acetic acid in deionized water. These solutions were provided as drinking water *ad libitum* beginning at Day 25 of age, designated as Day 0 of treatment.

The rats were fed a pelleted semipurified defined diet (Test Diet No. 5755, Purina Mills, Richmond, IN) containing 0.60% calcium, 0.40% phosphorus, and 2200 IU/kg vitamin D_3. The diet was chosen because it is considered nutritionally adequate (replete) for both growing and pregnant rats according to National Research Council (NRC) guidelines (NRC, 1978), but does not provide supplementary amounts of nutrients normally found in laboratory rat chow. The same production batch of diet was used throughout the study.

Prior to mating, weekly body weight and tail length of the female rats were measured beginning at initiation of exposure (Day 0). An adapted 25-cm ruler (1 mm scale) was used to measure rat tail length. Blood was taken by tail section at Days 0, 21, and 49 for blood lead analysis by atomic absorption spectrophotometry (Pruszkowska *et al.*, 1983). The blood samples were collected in 0.1-ml capillary pipets (Environmental Health Associates, Rochester, NY) from incisions made at the base of the tail after the tail had been cleaned with a 0.5% disodium ethylenediaminetetraacetate (EDTA) solution and rinsed with deionized water.

After 49 days of exposure, mating was initiated. Each female rat was taken when proestrous and mated overnight with a control male. Vaginal smears were read on the following morning. The day of appearance of sperm in the vaginal smears was designated as Day 0 of gestation. *Ad libitum* feeding conditions began at pregnancy (Gestation Day 0). The pregnant females were singly housed until Gestation Day 18. On Day 18, the pregnant females were transferred to baskets with wood chip bedding. Exposure conditions did not change during gestation. Six of the 48 rats found proestrous and which did not become pregnant after three mating attempts were removed from the study.

At parturition. At parturition, the number of pups in each litter was culled by random selection to eight. As shown in Fig. 1, 20 of 24 control dams had litters, and 22 of 24 lead-exposed dams had litters. Six dams each from the 250 and 1000 ppm lead groups were removed from lead exposure and given control drinking water. Six dams each from the control group were given 250 or 1000 ppm lead in drinking water. Exposure conditions for the remaining dams in the control, 250, and 1000 ppm groups were not changed. *Ad libitum* feeding conditions continued during lactation. Parturition study groups are shown in Fig. 1. They are: Group 1, control/control (0/0); Group 2, 250 ppm/control (250/0); Group 3, 1000 ppm/control (1000/0); Group 4, control/250 ppm (0/250); Group 5, control/1000 ppm (0/1000); Group 6, 250 ppm/250 ppm (250/250); and Group 7, 1000 ppm/1000 ppm (1000/1000).

Within 12 to 24 hr after parturition, Day 1 pup body weight of all pups (including culled pups) was measured, and gestation time (days to parturition) and gestation rate (number of dams with litters) were recorded.

After parturition. The dams were killed by asphyxiation with carbon dioxide on Day 21 of lactation, and the weanlings were killed with carbon dioxide 3 days later. During the 3 days, the weanlings were given the same drinking water previously received by their dams to assess the effect of lead on early weanling growth and mortality rate.

Body weight of the suckling pups were measured on Day 11 of lactation. At Day 21, blood was taken by tail section from the dams for blood lead analysis and determination of serum calcium and phosphorus by atomic absorption spectroscopy. The dams were then killed by asphyxiation with carbon dioxide, and both tibias were taken. The left tibia was used for determination of tibial ash weight and tibial lead (Wittmers *et al.*, 1981), phosphorus (Chen *et al.*, 1956), and calcium (Halloran and DeLuca, 1981). The right tibia was used for measurement of growth-plate width (see below).

Three days later, body weight and tail length of Day 24 weanlings were measured, and serum was taken by tail section for calcium and phosphorus measurement. The weanlings were then killed by asphyxiation with carbon dioxide, and tibias were taken for determination of tibial ash weight, lead, calcium, and phosphorus (left tibia), or for measurement of growth-plate width (right tibia).

The methods used for tibia specimen fixation, embedding, and sectioning are based on previously described procedures (Schenk *et al.*, 1984). Proximal tibia specimens were fixed in Millonig's fixative (Sheehan and Hrapchak, 1980) for 24 hr, dehydrated, and embedded in polymerized methyl methacrylate. Thick (approximately 0.5 mm) sagittal sections of

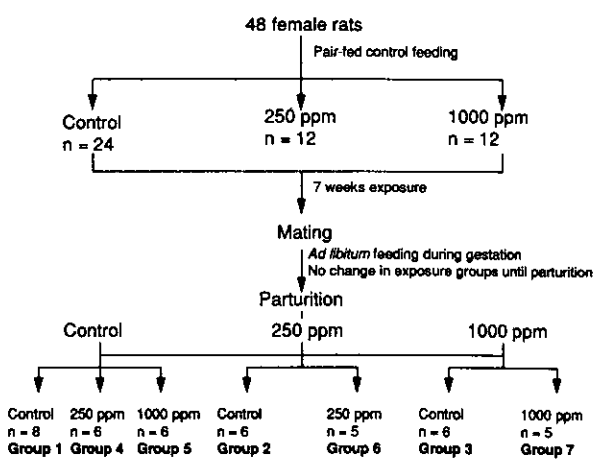

FIG. 1. Animals and exposure groups for assessment of the growth of lead-exposed female rats and skeletal development in their offspring.

the proximal tibial epiphysis were sectioned with a diamond-tipped saw to reach the growth plate and upper metaphyseal region. Sequential sagittal sections 7 μm thick were then taken with a sliding microtome fitted with a tungsten–carbide knife. Four sections were prepared from each specimen.

The embedded tibia sections were deplasticized with three 15-min changes of 2-methoxyethyl acetate and hydrated in graded ethanol solutions (70% for 5 min, 40% for 5 min, and deionized water for 1 hr). For examination of trabecular morphology in the metaphysis and growth-plate width measurement, the hydrated sections were stained using the Von Kossa reaction for phosphate with McNeal's tetrachrome counterstain, and growth-plate morphology was examined with Movat's stain for mineralized and nonmineralized cartilage (Schenk *et al.*, 1984). All photomicrographs were taken at 100× magnification with a camera-mounted microscope using a green light filter.

For determination of growth-plate width, five transversely equally spaced measurements were taken on each stained section with a calibrated eyepiece micrometer by light microscopy at 40× magnification. The measurements were taken along the longitudinal axis of the tibia from the first layer of germinal chondrocytes to the last layer of terminal chondrocytes. Preliminary analysis of variance (ANOVA) showed that there were no differences among the five growth-plate width measurements per section. Therefore, the mean of the five growth-plate width measurements was recorded as section growth-plate width.

Preliminary statistical analyses indicated that no sex-related effects were associated with lead exposure in this study. All data were pooled within each treatment group and expressed as the treatment group means ± standard error (SE). The effects of lead were evaluated across treatment groups by ANOVA with the Duncan–Bonner multiple range *t* test to determine significance ($p \leq 0.05$).

RESULTS

Prior to Parturition

Dam body weight, tail length, and blood lead (Table 1). No lead-related effects on pair-fed female rat body weight and tail length were observed. Body weights and tail lengths measured on Day 0 (exposure initiation) and on Day 49 are presented (Table 1). Blood lead levels in the 250 and 1000 ppm groups at Day 21 were higher ($p \leq 0.05$) than the blood lead levels in the same groups on Day 49.

At Parturition

Lead did not affect gestation time (average 22 days), gestation rate (20 litters of 24 controls, 11 litters of 12 in 250 ppm group, 11 litters of 12 in 1000 ppm group), number of live pups per litter (12.6 pups in control group, 13.2 pups in 250 ppm group, 12.2 pups in 1000 ppm group), or pup mortality (less than 1% mortality from Day 1 to 24 across all study groups). No abnormalities were evident at parturition.

After Parturition

Offspring body weight and dam blood lead (Table 2). Day 1 suckling body weight was not affected by lead. However, Day 11 suckling body weight was decreased ($p \leq 0.05$) in Group 7 (1000/1000). By Day 24, decreased ($p \leq 0.05$) weanling body weight was evident in Groups 5 (0/1000), 6 (250/250), and 7 (1000/1000). Mean maternal blood lead levels at termination (i.e., end of lactation) in Groups 5, 6, and 7 were 77.9 μg/dl or higher.

Serum calcium and phosphorus (Table 3). With continuous 1000 ppm lead exposure, serum calcium was reduced ($p \leq 0.05$) in Group 7 (1000/1000) dams, and both serum phosphorus and serum calcium were decreased ($p \leq 0.05$) in Group 7 weanlings. Although statistically significant, the changes were not substantial and the calcium and phosphorus levels in all groups were within expected serum calcium and phosphorus ranges for rats (Kaneko, 1989).

Tibial ash weight, calcium, and phosphorus (Table 4). There was no effect of lead on tibial ash weight or on weanling tibial calcium and phosphorus levels. However, tibial calcium and phosphorus levels were decreased ($p \leq 0.05$) in Group 6 (250/250) and Group 7 (1000/1000) dams.

Tibial lead, growth-plate width, and tail length (Table 5). Although dams in Groups 2 (250/0) and 3 (1000/0) were removed from lead at parturition, their tibial lead levels were much greater ($p \leq 0.05$) than those in dams exposed to lead only after parturition. In contrast, Groups 2 and 3 weanling tibial lead levels were much less ($p \leq 0.05$) than tibial lead levels in weanlings exposed to lead only after parturition. When measured on Day 24 of lactation,

TABLE 1
Dam Body Weight, Tail Length, and Blood Lead Prior to Mating

Group	Number prior to mating	Body weight (g)		Tail length (cm)		Blood lead (μg/dl)		
		Day 0	Day 49	Day 0	Day 49	Day 0	Day 21	Day 49
Control	24	63.9 (1.1)	217.9 (4.2)	10.6 (0.3)	19.8 (0.3)	3.6 (0.7)	2.3 (0.6)	2.7 (0.6)
250 ppm	12	64.3 (1.3)	222.2 (8.1)	10.5 (0.3)	20.0 (0.1)	2.9 (0.8)	50.0 (1.8)*	39.9 (3.5)*,**
1000 ppm	12	63.3 (1.0)	224.0 (7.7)	10.5 (0.2)	19.9 (0.2)	3.9 (0.5)	93.4 (8.6)*	73.5 (9.3)*,**

Note. Values are means (±SE).
* Significantly ($p < 0.05$) greater than control.
** Significantly ($p \leq 0.05$) less than Day 21 blood lead.

TABLE 2
Number of Litters, Offspring Body Weight, and Dam Blood Lead

Group	Number of litters	Offspring body weight (g) Suckling (Day 1)	Offspring body weight (g) Suckling (Day 11)	Offspring body weight (g) Weanling (Day 24)	Dam blood lead* at euthanization (μg/dl)
1	8	5.74 (0.2)	23.6 (0.4)	59.4 (1.9)	1.6 (0.4)
2	6	5.51 (0.1)	23.1 (0.6)	60.9 (0.9)	17.5 (1.6)
3	6	5.82 (0.2)	23.9 (1.4)	63.2 (3.0)	40.8 (7.1)
4	6	5.68 (0.1)	22.2 (1.6)	53.5 (2.8)	71.7 (28.6)
5	6	6.11 (0.3)	23.0 (1.4)	50.9 (3.8)**	119.7 (17.4)
6	5	5.52 (0.2)	21.4 (1.7)	49.9 (1.5)**	77.9 (16.9)
7	5	5.69 (0.1)	20.7 (0.8)**	49.3 (1.2)**	114.3 (8.8)

Note. Values are means (±SE).
* Group 2 to 7 blood lead levels were significantly ($p \leq 0.05$) greater than control.
** Significantly ($p \leq 0.05$) less than control.

weanling tail length was decreased ($p \leq 0.05$) in Groups 5 (0/1000), 6 (250/250), and 7 (1000/1000).

No alteration in growth-plate width was apparent in the dams. However, increased ($p \leq 0.05$) growth-plate width was observed in weanlings continuously exposed to 1000 ppm lead (Group 7).

Growth-plate and metaphyseal morphology. Tibial metaphyseal trabeculae below the growth plate appeared to be wider in dams in Groups 6 (250/250) and 7 (1000/1000), but alterations in growth-plate morphology in the dams were not apparent. In the weanlings, control group growth-plate regions contained orderly columns of growth-plate chondrocytes (Fig. 2) and generally longitudinal metaphyseal trabeculae with few branches (Fig. 3), whereas Group 7 (1000/1000) weanling growth-plate regions were characterized by disrupted orientation of growth-plate chondrocytes, reduced mineralization of the cartilage matrix (Fig. 4), and increased branching with possibly increased width of the metaphyseal trabeculae (Fig. 5). Increased branching of metaphyseal trabeculae was also evident in weanlings in Groups 5 (0/1000) and 6 (250/250). A reduction in the number of hypertrophying chondrocytes and an increase in the amount of nonmineralized cartilage were judged to be evident in Group 7 weanling growth plates.

DISCUSSION

The physiological interaction of lead and food intake has received attention in studies of the mechanisms of lead-related effects on growth (Hammond *et al.*, 1989). Results from pair feeding in this study and results from previous studies (Hamilton *et al.*, 1994; Hammond *et al.*, 1989) suggest that lead-related effects on food intake may be a component of the effects of lead on growth rate. It has been shown (Hamilton *et al.*, 1994) that with weanling rats fed *ad libitum,* reduced growth rate (body weight and tail length) asso-

TABLE 3
Serum Calcium and Phosphorus at Euthanization

Group	Dams Serum calcium (mg/dl)	Dams Serum phosphorus (mg/dl)	Weanlings Serum calcium (mg/dl)	Weanlings Serum phosphorus (mg/dl)
1	11.4 (0.3)	8.2 (0.6)	11.7 (0.1)	11.5 (0.1)
2	11.0 (0.9)	8.4 (0.8)	11.2 (0.1)	11.9 (0.1)
3	11.2 (0.1)	8.7 (0.8)	11.5 (0.1)	10.9 (0.1)
4	11.6 (0.3)	9.3 (1.8)	11.0 (0.2)	10.6 (0.4)
5	11.2 (0.4)	7.9 (0.6)	10.9 (0.1)	10.8 (0.2)
6	11.0 (0.4)	7.9 (0.7)	10.9 (0.1)	10.6 (0.1)
7	10.4 (0.3)*	8.1 (0.5)	10.6 (0.2)*	10.1 (0.1)*

Note. Values are means (±SE).
* Significantly ($p < 0.05$) less than control.

TABLE 4
Tibial Ash, Calcium, and Phosphorus

Group	Dams			Weanlings		
	Ash (mg)	Ca (mg/g ash)	P (mg/g ash)	Ash (mg)	Ca (mg/g ash)	P (mg/g ash)
1	243.1 (10.1)	435.8 (20.4)	195.4 (12.5)	34.1 (4.1)	421.9 (15.3)	238.8 (11.3)
2	242.2 (13.1)	383.2 (22.6)	175.4 (11.6)	38.0 (3.3)	384.8 (15.5)	209.8 (18.6)
3	253.2 (14.2)	384.2 (20.7)	174.6 (14.3)	38.5 (4.3)	378.9 (17.3)	207.9 (16.8)
4	241.1 (15.6)	401.5 (36.9)	190.8 (13.2)	36.3 (6.2)	407.1 (14.7)	216.0 (16.5)
5	250.1 (13.7)	383.9 (26.4)	187.9 (14.6)	33.2 (4.2)	441.4 (18.2)	202.0 (25.2)
6	260.4 (18.2)	355.1 (28.1)*	161.5 (13.8)*	29.1 (5.1)	463.9 (13.2)	241.4 (17.5)
7	262.4 (17.5)	359.5 (21.4)*	163.1 (14.4)*	30.4 (5.6)	451.4 (21.1)	216.0 (14.1)

Note. Values are means (±SE).
* Significantly ($p \leq 0.05$) less than control.

ciated with up to 1000 ppm lead in drinking water was maintained because of a high correlation between growth and food intake, *not* because of a continuous negative effect of lead directly on growth. In the current study, weanling growth (i.e., of females prior to mating) was not altered by up to 1000 ppm lead in drinking water when food intake was controlled by pair feeding a nutritionally adequate diet.

Blood lead concentrations in the female rats prior to mating were significantly and markedly higher after 21 days of lead exposure starting at weaning than after 49 days of lead exposure (Table 1). This seemingly anomalous observation is probably a consequence of the sharp reduction in fractional absorption of lead from the gastrointestinal tract that follows weaning in rats (Kostial *et al.*, 1978).

Absence of lead-related effects on gestation time, gestation rate, litter size, and offspring mortality in this study is consistent with results from a previous study in rats with continuous lead exposure (Grant *et al.*, 1980). However, absence of effects on reproduction does not imply an absence of effects on early postnatal development. Continuous lead exposure in drinking water caused a greater decrease in offspring body weight than lead exposure only prior to parturition or lead exposure only after parturition. The contribution of *in utero* exposure to postnatal growth reduction has also been observed in lead-exposed human infants. Combination of *in utero* exposure and lead exposure after parturition was associated with more severe reductions in statural growth and body weight gain in infants than *in utero* exposure or postparturition lead exposure alone (Shukla *et al.*, 1989).

Reduction in offspring body weight began at relatively high maternal blood lead levels, equal to or greater than 77.9 μg/dl at the end of lactation. Reduction in offspring body weight at relatively high maternal blood lead levels has been observed in other studies with rats, although earlier and at lower administered doses. Offspring body weight was reduced beginning at Day 1 of age in a chronic study with 50 or 250 ppm lead in drinking water (Grant *et al.*, 1980). Average maternal blood lead concentrations in that study were 50 μg/dl in the 50 ppm group and 99 μg/dl in the

TABLE 5
Tibial Lead, Growth-Plate Width, and Weanling Tail Length

Group	Tibial lead (μg/g ash)		Growth-plate width (μm)		Weanling Day 24 tail length (cm)
	Dams	Weanlings	Dams	Weanlings	
1	38.3 (5.5)	41.1 (3.9)	82.5 (8.5)	572.8 (12.3)	9.77 (0.15)
2	786.4 (15.7)*	97.4 (8.9)*	78.3 (6.6)	571.7 (13.3)	10.14 (0.06)
3	981.9 (19.6)*	163.2 (10.1)*	83.4 (7.9)	554.7 (13.8)	10.12 (0.27)
4	273.4 (10.6)*	1932.8 (24.2)*	76.3 (9.2)	558.7 (19.6)	9.95 (0.22)
5	403.6 (15.4)*	3196.9 (100.2)*	87.6 (10.2)	581.4 (9.6)	9.56 (0.13)*
6	970.0 (26.8)*	2096.6 (97.1)*	89.1 (17.3)	598.1 (15.5)	9.43 (0.04)*
7	1101.9 (30.2)*	2950.0 (66.4)*	83.9 (20.5)	630.9 (8.3)*	9.46 (0.06)*

Note. Values are means (±SE).
* Significantly ($p \leq 0.05$) different from control.

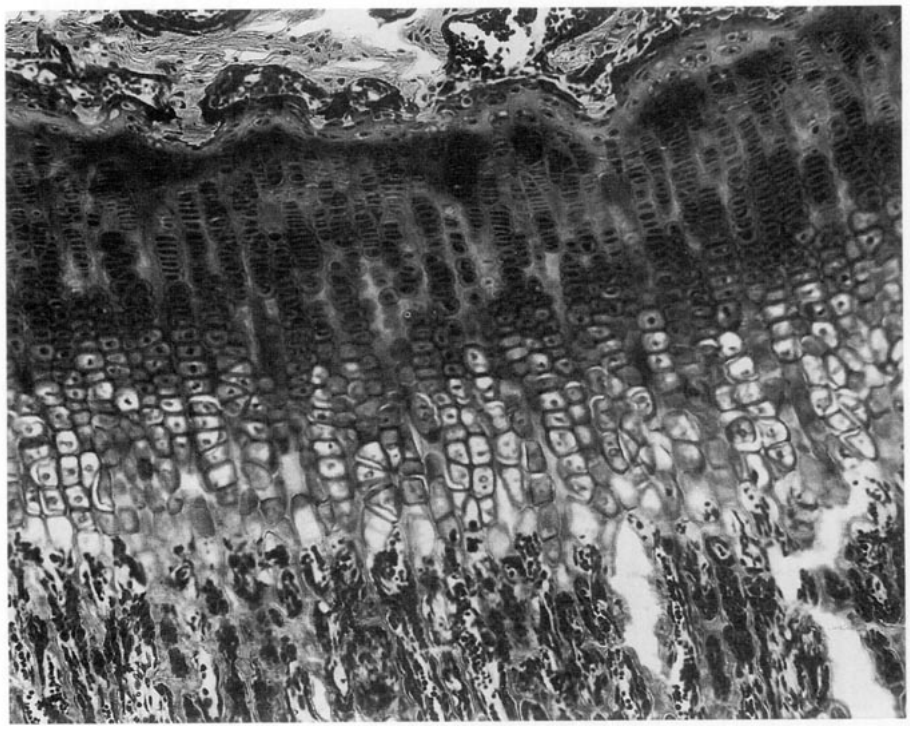

FIG. 2. Light microscopy of the proximal tibial growth-plate region (Movat's stain; 100× magnification) from a Group 1 (control) Day 24 weanling offspring.

250 ppm group at parturition, so that they were in the range associated in the current study with higher drinking water lead concentrations. The diet (as defined in Kimmel *et al.*, 1980) contained somewhat lower levels of calcium, phosphorus, and other nutrients than the diet used here and lower than those recommended for rats (NRC, 1978). Lowering dietary calcium or phosphorus will increase lead absorption, milk lead transfer, and offspring bone lead accumulation (Quarterman *et al.*, 1973; Mahaffey, 1981). Irrespective of possible diet-related influences on lead absorption, however, the current and previous (Grant *et al.*, 1980) studies together indicate that *in utero* exposure combined with postnatal exposure can cause substantial reductions in early postnatal growth. It is noteworthy that maternal blood lead concentration on Day 21 after parturition does not correlate particularly well with the magnitude of reduction in offspring body weight, but instead tends to reflect recent maternal exposure (Table 2). The pattern and magnitude of maternal lead ingestion during the total period from conception to Day 24 postparturition are the determinants of weanling body weight.

It has been suggested that maternal skeletal lead may be mobilized in conditions, such as lactation, in which bone demineralization may occur (Silbergeld, 1991). As shown in a previous study (Keller and Doherty, 1980), blood lead in the continuously lead-exposed dams at the end of lactation was higher than blood lead measured prior to mating. The continuous increase in blood lead levels would be expected in these females, who were still growing during the course of pregnancy and lactation and were therefore actively depositing lead in mineralizing bone. Continuous interchange between blood and bone lead causes the two measures to track each other. Other contributors to the observed increase in blood lead concentrations in the continuously lead-exposed dams could have been increased lead absorption (Kostial and Momcilovic, 1974) and/or increased maternal fluid consumption as well as skeletal lead remobilization during lactation.

Lead exposure prior to parturition caused greater maternal tibial lead accumulation than lead exposure after parturition. This observation is consistent with a decline in maternal growth rates during the course of the study, as well as with the lengths of exposure. Those dams whose controlled exposure covered only lactation were older, and therefore growing less rapidly, at the end of their exposure period than the dams exposed only before and during pregnancy. They also had been exposed to lead for a shorter time (21 rather than 70 days). Therefore, it is to be expected that relatively less tibial lead would have accumulated during lactation than before and during pregnancy. Other factors may also contribute to the difference in rates of lead uptake by the tibia. The female rat skeleton will increase in mineral content during gestation (Quan-Sheng and Miller, 1989), but skeletal demineralization occurs in the rat during lacta-

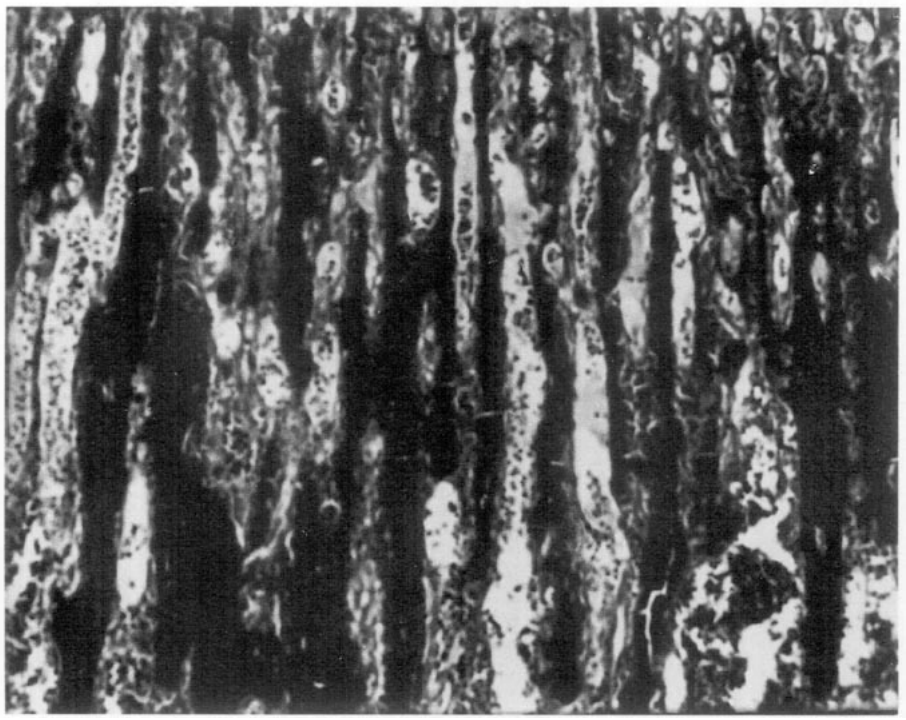

FIG. 3. Light microscopy of the proximal tibial metaphyseal region (Von Kossa with McNeal's tetrachrome stain; 100× magnification) from a Group 1 (control) Day 24 weanling offspring.

tion (Halloran and DeLuca, 1980). Through increasing demands for calcium, lactation is likely to increase mobilization of bone lead. Bone lead mobilization has been demonstrated *in vitro* with low concentrations of calcium in a bone culture medium (Rosen and Wexler, 1977). It is possible that by mechanisms such as these, the net rate of maternal tibial lead accumulation may be reduced during lactation.

The timing of maternal exposure also had an impact on offspring tibial lead levels. Dams exposed to lead prior to parturition had higher tibial lead levels than dams exposed to lead only after parturition. However, in the case of the pups, exposure prior to parturition had a much smaller impact on tibial lead accumulation than exposure after parturition (Table 5). Lead transferred to the sucklings via maternal milk, as well as possible lead from drinking water during late stages of lactation, was by far the most important source of weanling tibial lead in this study (Table 5). Indeed, maternal blood lead concentration, also determined largely by postpartum exposure, correlates almost perfectly with weanling tibial bone lead in the four groups exposed after parturition (Groups 4–7). Postnatal bone mineralization is very rapid in rats (Baron *et al.*, 1984). When rapid bone growth is combined with high fractional absorption of lead from the gastrointestinal tract, it can be predicted that lactation should be a period of particularly rapid incorporation of orally administered lead into bone. In confirmation of this expectation, comparison of total tissue levels with the amount of lead ingested has demonstrated that femurs of weanling rats exposed to lead in drinking water contained a much greater fraction of the ingested lead than femurs of adult rats (Rader *et al.*, 1981). In addition, it has been reported that a greater amount of maternal lead is transferred in milk to mouse offspring during lactation than is transferred transplacentally during gestation (Keller and Doherty, 1980).

The statistically significant reduction in maternal and weanling serum calcium levels caused by continuous 1000 ppm lead exposure (Table 3) were slight and probably not biologically significant (Kaneko, 1989). Up to 1000 ppm lead in drinking water had not been expected to alter weanling serum calcium or phosphorus levels because alterations in milk volume and nutrient content were not observed below approximately 1300 ppm lead (0.2% lead acetate) in drinking water in lactating rats (Bornschein *et al.*, 1977).

More substantial changes in calcium and phosphorus were recorded in maternal tibia (Table 4). Continuous exposure to lead may have accelerated maternal bone calcium and phosphorus loss through actions of lead on vitamin D-dependent bone formation and/or gastrointestinal uptake of calcium. 1,25-Dihydroxyvitamin D_3 may be essential for normal maternal bone formation rates during pregnancy and lactation (Marie *et al.*, 1986), and lead has been shown to reduce 1,25-dihydroxyvitamin D_3 levels and in-

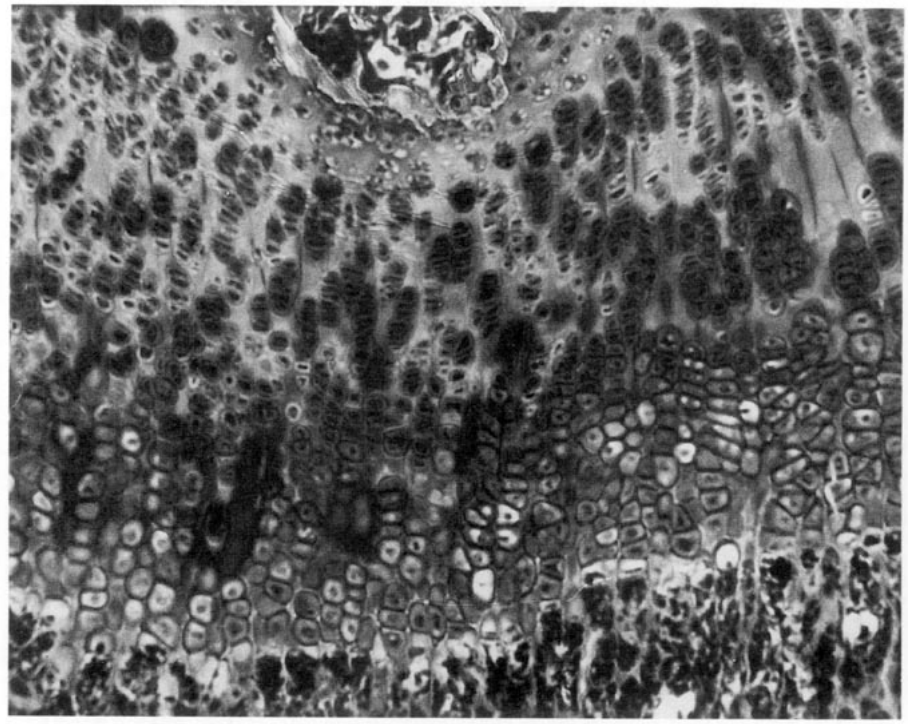

FIG. 4. Light microscopy of the proximal tibial growth-plate region (Movat's stain; 100× magnification) from a Day 24 weanling offspring continuously exposed to 1000 ppm lead (Group 7).

hibit 1,25-dihydroxyvitamin D_3-dependent intestinal calcium uptake (Gruden, 1975; Smith *et al.*, 1981).

Reduced maternal tibial calcium and phosphorus was not associated with alterations in offspring tibial calcium and phosphorus concentrations, which were normal in the lead-exposed weanlings (Table 4), indicating that adequate calcium and phosphorus had been provided to the offspring during gestation and lactation. However, although weanling total tibial calcium and phosphorus levels were not affected, bone mineral utilization was altered by lead. Increased tibial growth-plate width, indicative of skeletal growth retardation (Greenspan *et al.*, 1949), was apparent in those weanlings continuously exposed to 1000 ppm lead. Increased growth-plate width was consistently associated with altered chondrocyte orientation and decreased cartilage matrix mineralization. The nature of these changes is also suggestive of growth retardation. Decreased tail length, possibly indicative of lead-related effects on tail vertebrae, was noted in weanlings continuously exposed to lead or exposed to 1000 ppm lead after parturition. Increased vertebral growth-plate widths have been reported in lead-exposed dogs (Stowe *et al.*, 1973). It is possible that the decreased statural growth seen in lead-exposed infants (Shukla *et al.*, 1989) may be associated with lead-related effects on vertebral and long bone growth plates.

The current study does not support a hypothesis that a lead-related maternal deficiency in vitamin D, if it occurred, inhibited weanling growth-plate development. Altered skeletal development seen in rat offspring from vitamin D-deficient dams is caused by a marked reduction in milk production (Mathews *et al.*, 1986). The proportionality of maternal blood lead and weanling tibial lead in the continuously exposed groups suggests that there was little or no effect of lead on milk production in the current study nor would any have been expected in view of Bornschein *et al.*'s (1977) observation that concentrations up to approximately 1300 ppm lead (0.2% lead acetate) in drinking water did not alter milk volume in lactating rats. Growth-plate development may have been altered through mechanisms local to the growth plate, possibly including effects on the process of growth-plate chondrocyte maturation, required for normal long bone growth and mineralization (Hunziker, 1988). This study provides a site-specific initial morphologic assessment of bone lead toxicity. Measurements based on quantitative histomorphometry (Hunziker *et al.*, 1987; Baron *et al.*, 1984) could more precisely define the dose–response of lead in growing bone.

Increased branching of Von Kossa stain-positive metaphyseal trabeculae was observed in weanlings with continuous exposure or with 1000 ppm postparturition exposure, and wider trabeculae were observed in dams with continuous exposure. Although weanling tibial calcium and phosphorus analyses did not suggest altered total tibial mineralization, lead may have affected trabecular development by

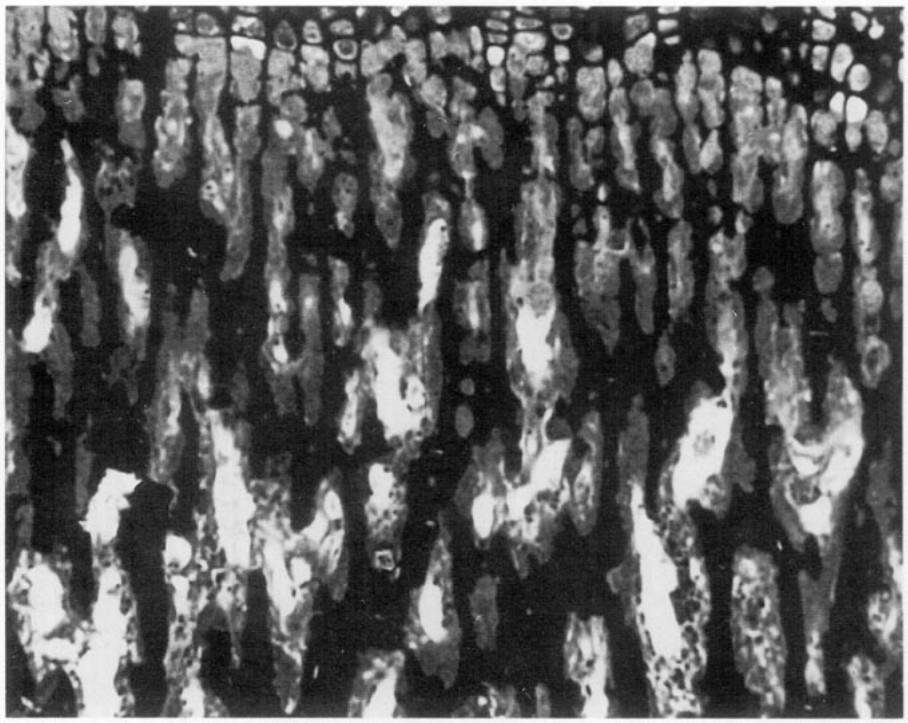

FIG. 5. Light microscopy of the proximal tibial metaphyseal region (Von Kossa with McNeal's tetrachrome stain; 100× magnification) from a Day 24 weanling offspring continuously exposed to 1000 ppm lead (Group 7).

altering resorption and mineralization. Impaired resorption of metaphyseal trabeculae in the presence of increased numbers of osteoclasts has been observed in lead-exposed rats (Eisenstein and Kawanoue, 1975; Hsu *et al.*, 1973). However, it should be noted that the Von Kossa stain involves the reaction of silver ions with phosphate-based (e.g., hydroxyapatite) salts (Schenk *et al.*, 1984) and will not distinguish between sites of lead hydroxyapatite deposition and sites of calcium hydroxyapatite deposition (McClure, 1980). Lead can induce calcium deposition (McClure, 1980; Gabbiani *et al.*, 1970) and can deposit in bone as lead hydroxyapatite (Verbeeck *et al.*, 1981). Lead fragments may cause abnormal mineralization in bone (Windler *et al.*, 1978). Therefore, the altered trabecular histomorphology seen in the weanlings and dams at high-lead exposure may be the result of the combined effects of lead deposition, lead-induced calcium deposition, and inhibition of resorption.

Growth-plate activity (e.g., rate of chondrocyte production and mineral turnover) is reported to be substantially increased in pregnant rats and to drop to prepregnancy levels by the end of lactation (Redd *et al.*, 1984). Although no lead-related effects on growth-plate morphology were observed in the dams in the present study, it is possible that they occurred but were reversible by the end of lactation. Although trabecular widths appeared to be increased, total tibial calcium and phosphorus levels were significantly reduced in dams continuously exposed to lead, indicating that lead exposure had accelerated maternal bone mineral loss during lactation.

The effects of lead on the dams in this study were clearly different from its effects on their offspring. Given an experimental design that controlled for food intake, lead exposure from weaning onward had no effect on female rat body weight. After pregnancy and lactation, maternal blood lead levels tended to reflect largely recent exposure (i.e., exposure during lactation), although a significant influence of bone lead stores on blood lead concentration was also apparent. Maternal tibial lead reflected exposure during both pregnancy and lactation. Accumulation of lead in weanling bone was determined largely by postnatal exposure; i.e., lead was most rapidly incorporated into bone during the period when the offspring were growing rapidly and were absorbing a large fraction of the lead present in the mother's milk. Lead dose-dependent reductions in maternal tibial calcium and phosphorus levels suggested the possibility of acceleration by lead of maternal bone calcium and phosphorus loss during gestation and/or lactation. Weanling total tibial calcium and phosphorus levels were not affected by lead exposure, demonstrating that adequate quantities of bone minerals were made available during lactation for bone formation, perhaps at the expense of maternal calcium and phosphorus stores. Nonetheless, weanling bone mineral utilization was altered, as demonstrated by disrup-

tions in growth-plate morphology and in metaphyseal region effects reported in other mammalian species.

Both prenatal and postnatal lead exposure contributed to a dose-related reduction in growth rate and body weight of weanling offspring; *in utero* lead exposure enhanced lead-related growth retardation when exposure was continued postnatally. Although the mechanisms of these effects are not known, the results suggest that local effects on growth-plate chondrogenesis and metaphyseal mineralization may be involved. An ectopic bone induction method (Urist, 1965; Muthukumaran and Reddi, 1985) has been used to simulate regions of bone growth where chondrogenesis is followed by cartilage mineralization, and the local effects of lead on ectopic bone chondrogenesis and mineralization will be described in a future publication.

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REFERENCES

Anderson, C., and Danylchuk, K. D. (1980). Haversian bone-remodelling rates in the beagle after cessation of exposure to chronic low doses of lead. *J. Environ. Pathol. Toxicol.* **3,** 413–422.

Angle, C. R., and Kunzelman, D. R. (1989). Increased erythrocyte protoporphyrins and blood lead—A pilot study of childhood growth patterns. *J. Toxicol. Environ. Health* **26,** 149–156.

Arsenault, A. L., and Hunziker, E. B. (1988). Electron microscopic analysis of mineral deposits in the calcifying epiphyseal growth plate. *Calcif. Tissue Int.* **42,** 119–126.

Baron, R., Tross, R., and Vignery, A. (1984). Evidence of sequential remodeling in rat trabecular bone: Morphology, dynamic histomorphometry, and changes during skeletal maturation. *Anat. Rec.* **208,** 137–145.

Bornschein, R. L., Fox, D. A., and Michaelson, I. A. (1977). Estimation of daily exposure in neonatal rats receiving lead via dam's milk. *Toxicol. Appl. Pharmacol.* **40,** 577–587.

Chen, P. S., Toribara, T. Y., and Warner, H. (1956). Microdetermination of phosphorus. *Anal. Chem.* **11,** 1756–1758.

Eisenstein, R., and Kawanoue, S. (1975). The lead line in bone—A lesion apparently due to chondroclastic indigestion. *Am. J. Pathol.* **80,** 309–316.

Gabbiani, G., Tuchweber, B., and Perrault, G. (1970). Studies in the mechanism of metal-induced soft tissue calcification. *Calcif. Tissue Res.* **6,** 20–31.

Grant, L. D., Kimmel, C. A., West, G. L., Martinez-Vargas, C. M., and Howard, J. L. (1980). Chronic low-level lead toxicity in the rat. II. Effects on postnatal physical and behavioral development. *Toxicol. Appl. Pharmacol.* **56,** 42–58.

Greenspan, F. S., Li, C. H., Simpson, M. E., and Evans, H. M. (1949). Bioassay of hypophyseal growth hormone: The tibia test. *Endocrinology* **45,** 455–463.

Gruden, N. (1975). Lead and active calcium transfer through the intestinal wall in rats. *Toxicology* **5,** 163–166.

Halloran, B. P., and DeLuca, H. F. (1980). Skeletal changes during pregnancy and lactation in rats: The role of vitamin D. *Endocrinology* **107,** 1923–1929.

Halloran, B. P., and DeLuca, H. F. (1981). Effect of vitamin D deficiency on skeletal development during early growth in the rat. *Arch. Biochem. Biophys.* **209,** 7–14.

Hamilton, J. D., O'Flaherty, E. J., Ross, R., Shukla, R., and Gartside, P. S. (1994). Effects of lead exposure on maternal and fetal growth in rats. *Environ. Res.* **64,** 53–64.

Hammond, P. B., Chernausek, S. D., Succop, P. A., Shukla, R., and Bornschein, R. L. (1989). Mechanism by which lead depresses linear and ponderal growth in weanling rats. *Toxicol. Appl. Pharmacol.* **99,** 474–486.

Hsu, F. S., Krook, L., Shively, J. N., Duncan, J. R., and Pond, W. G. (1973). Lead inclusion bodies in osteoclasts. *Science* **181,** 447–448.

Hunziker, E. B. (1988). Growth plate structure and function. *Pathol. Immunopathol. Res.* **7,** 9–13.

Hunziker, E. B., Schenk, R. K., and Cruz-Orive, L. M. (1987). Quantitation of chondrocyte performance in growth-plate cartilage during longitudinal bone growth. *J. Bone Surg.* **69A,** 162–173.

Kaneko, J. J. (1989). *Clinical Biochemistry of Domestic Animals,* Appendix VIII, pp. 892–897. Academic Press, New York.

Keller, C. A., and Doherty, R. A. (1980). Bone lead mobilization in lactating mice and transfer to suckling offspring. *Toxicol. Appl. Pharmacol.* **55,** 220–228.

Kennedy, G. L., Arnold, D. W., and Calandra, J. C. (1975). Teratogenic evaluation of lead compounds in mice and rats. *Food Cosmet. Toxicol.* **13,** 629–635.

Kimmel, C. A., Grant, L. D., Sloan, C. S., and Gladen, B. C. (1980). Chronic low-level lead toxicity in the rat. I. Maternal toxicity and perinatal effects. *Toxicol. Appl. Pharmacol.* **56,** 28–41.

Kostial, K., Kello, D., Jugo, S., Rabar, I., and Maljkovic, T. (1978). Influence of age on metal metabolism and toxicity. *Environ. Health Perspect.* **25,** 81–86.

Kostial, K., and Momcilovic, B. (1974). Transport of lead 203 and calcium 47 from mother to offspring. *Arch. Environ. Health* **29,** 28–30.

Mahaffey, K. R. (1981). Nutritional factors in lead poisoning. *Nutr. Rev.* **39,** 353–362.

Marie, P. J., Cancela, L., Le Boulch, N., and Miravet, L. (1986). Bone changes due to pregnancy and lactation: Influence of vitamin D status. *Am. J. Physiol.* **251,** E400–E406.

Mathews, C. H. E., Brommage, R., and DeLuca, H. F. (1986). Role of vitamin D in neonatal skeletal development in rats. *J. PHysiol.* **250,** E725–E730.

McClain, R. M., and Becker, B. A. (1975). Teratogenicity, fetal toxicity, and placental transfer of lead nitrate in rats. *Toxicol. Appl. Pharmacol.* **31,** 72–82.

McClure, J. (1980). The production of heterotopic calcification by certain chemical salts. *J. Pathol.* **131,** 21–33.

Mooty, J., Ferrand, C. J., and Harris, P. (1975). Relationship of diet to lead poisoning in children. *Pediatrics* **55,** 636–639.

Muthukaran, N., and Reddi, A. H. (1985). Bone matrix-induced local bone induction. *Clin. Orthop.* **200,** 159–164.

National Research Council (NCR) (1978). Nutrient requirements of the rat. In *Nutrient Requirements of Laboratory Animals,* pp. 7–37. National Academy of Sciences, Washington DC.

Nordberg, G. F., Mahaffey, K. R., and Fowler, B. A. (1991). Introduction

and summary. International workshop on lead in bone: Implications for dosimetry and toxicology. *Environ. Health Perspect.* **91,** 3–7.

Park, E. A., Jackson, D., Goodwin, T. C., and Kajdi, L. (1933). X-ray shadows in growing bones produced by lead: Their characteristics, cause, anatomical counterpart in the bone and differentiation. *J. Pediatr.* **3,** 265–298.

Pearl, M., and Boxt, L. M. (1980). Radiographic findings in congenital lead poisoning. *Radiology* **136,** 83–84.

Pruszkowska, E., Carnick, G. R., and Slavin, W. (1983). Blood lead determination with the platform furnace technique. *At. Spectrosc.* **4,** 59–61.

Quan-Sheng, D., and Miller, S. C. (1989). Calciotrophic hormone levels and calcium absorption during pregnancy in rats. *Am. J. Physiol.* **257,** E118–E112.

Quarterman, J., Morrison, J. N., and Carey, L. F. (1973). The influence of dietary calcium and phosphate on lead metabolism. In *Trace Substances in Environmental Health* (D. D. Hemphill, Ed.), Vol. 7, pp. 347–352. Univ. Missouri Press.

Rader, J. I., Peeler, J. T., and Mahaffey, K. R. (1981). Comparative toxicity and tissue distribution of lead acetate in weanling and adult rats. *Environ. Health Perspect.* **42,** 187–195.

Redd, E. H., Miller, S. C., and Jee, W. S. S. (1984). Changes in endochondral bone elongation rates during pregnancy and lactation during rats. *Calcif. Tissue Int.* **36,** 697–701.

Rosen, J. F., and Wexler, E. E. (1977). Studies of lead transport in bone organ culture. *Biochem. Pharmacol.* **26,** 650–652.

Schenk, R. K., Olah, A. J., and Herrmann, W. (1984). Preparation of calcified tissues for light microscopy. In *Methods of Calcified Tissue Preparation* (G. R. Dickson, Ed.), pp. 1–56. Elsevier, New York.

Schwartz, J., Angle, C., and Pitcher, H. (1986). Relationship between childhood blood lead levels and stature. *Pediatrics* **77,** 281–288.

Sheehan, D. C., and Hrapchak, B. B. (1980). Carson's modified Millonig's phosphate-buffered formalin. In *Theory and Practice of Histotechnology,* 2nd ed., p. 46. Lipshaw/Mosby, St. Louis, MO.

Shukla, R., Bornschein, R. L., Dietrich, K. N., Buncher, C. R., Berger, O., Hammond, P. B., and Succop, P. A. (1989). Fetal and infant lead exposure: Effects on growth and stature. *Pediatrics* **84,** 604–612.

Silbergeld, E. K. (1991). Lead in bone: Implications for toxicology during pregnancy and lactation. *Environ. Health Perspect.* **91,** 63–70.

Smith, C. M., DeLuca, H. F., Tanaka, Y., and Mahaffey, K. R. (1981). Effects of lead ingestion on functions of vitamin D and its metabolites. *J. Nutr.* **111,** 1321–1329.

Stowe, H. D., Goyer, R. A., Krigman, M. M., Cson, M. W., and Cates, M. (1973). Experimental oral lead toxicity in young dogs. *Arch. Pathol.* **95,** 106–116.

Urist, M. R. (1965). Bone formation by autoinduction. *Science* **150,** 893–896.

Verbeeck, R. M. H., Lassuyt, C. J., Heijligers, H. J. M., Driessens, F. C. M., and Vrolijk, J. W. G. A. (1981). Lattice parameters and cation distribution of solid solutions of calcium and lead hydroxyapatite. *Calcif. Tissue Int.* **33,** 243–247.

Windler, E. C., Smith, R. B., Bryan, W. J., and Woods, G. W. (1978). Lead intoxication and traumatic arthritis of the hip secondary to retained bullet fragments. *J. Bone J. Surg.* **60A,** 254–255.

Wittmers, L. E., Alich, A., and Aufderheide, A. C. (1981). Lead in bone. I. Direct analysis for lead in milligram quantities of bone ash by graphite furnace atomic absorption spectrophotometry. *Am. J. Clin. Pathol.* **75,** 80–85.