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Effects of Cadmium, Calcium, Age and Parity on Bone Mineral, Density and Strength in Female Rats

Beverley F. Hammond
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To the Graduate Council:

I am submitting herewith a dissertation written by Beverley F. Hammond entitled "Effects of Cadmium, Calcium, Age and Parity on Bone Mineral, Density and Strength in Female Rats." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

Jane R. Savage, Major Professor

We have read this dissertation and recommend its acceptance:

Polly G. Martin, Frances E. Andrews, Marjorie P. Penfield, Bert H. Erickson

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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EFFECTS OF CADMIUM, CALCIUM, AGE AND PARITY ON BONE MINERAL, DENSITY AND STRENGTH IN FEMALE RATS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Beverley F. Hammond

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ABSTRACT

Weanling female rats were fed diets containing one of three levels of Ca (0.3, 0.6 or 0.9%) and one of four levels of Cd (0, 1, 5 or 10 ppm) in the drinking water. One half of each group was bred first as adolescents (55 days) and the other half as mature (110 days) females. Approximately 10 animals from each group were sacrificed after the first pregnancy and the remaining animals after the fourth pregnancy. Reproductive performance, plasma and bone Ca and P and bone density and strength were measured.

After the first pregnancy, offspring of dams treated with 5 or 10 ppm Cd were smaller at birth than offspring of dams treated with 0 or 1 ppm Cd. After the fourth pregnancy, the decreased birth weight was evident only in offspring of dams treated with 10 ppm Cd. Offspring of dams fed 5 or 10 ppm Cd or the 0.3% Ca diet had decreased weaning weight regardless of parity. A 0.3% Ca diet superimposed upon a 5 or 10 ppm Cd intake decreased weaning weight of the male offspring after the first, but not the fourth, pregnancy with the offspring of adolescent dams affected more than those of mature dams. Offspring of dams fed the 0.9% Ca diet did not differ in weaning weight from the offspring of dams fed the 0.6% Ca diet.

Cadmium treatment had no effect on the plasma Ca or the Ca-P ratio. At Cd levels of 5 or 10 ppm the plasma P was increased. The 0.3% Ca diet depressed the plasma Ca and the 0.9% Ca diet elevated the plasma Ca and depressed the plasma P when compared to the 0.6%

diet. Parity did not affect plasma Ca but, after four pregnancies, plasma P was decreased. Plasma Ca of mature dams was higher than that of adolescent dams but plasma P was unaffected. Bone mineral, density and strength were decreased by the 0.3% Ca diet especially when Cd levels reached 10 ppm. Increasing dietary Ca above normal increased femur Ca of dams fed 1 ppm Cd but did not increase the Ca of the femur of dams given higher levels of Cd. After the first pregnancy, femur Ca of mature dams was greater than that of adolescent dams. After the fourth pregnancy, femurs of mature dams were less strong than those of adolescent dams; however, the density was the same. Increasing dietary Ca above 0.6% lessened the detrimental effects of 5 ppm Cd ingestion on bone density. Mature dams were less affected by the 0.3% Ca 10 ppm Cd treatment than were adolescent dams.

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CHAPTER I

INTRODUCTION

The loss of mineral from bone, which starts in the fourth decade of life and accelerates after the menopause, is a serious problem for many women. This demineralization of bone appears to take place regardless of dietary calcium (Ca) intake. However, the loss seems to be greater when the Ca intake is low chronically (1).

Demographic studies indicate there is an increase in the number of pregnancies in young girls in the 12-14 year old age group (2, 3). These girls are still in the growth stage and have not attained skeletal maturity. The intake of Ca at this age may be only a fraction of that needed for bone mineralization during the period of both fetal and maternal growth. Survey data indicate that the earlier the first pregnancy occurs, the more children the woman ultimately has (4). Numerous pregnancies in these very young girls, whether or not accompanied by the lactation period, may affect seriously the mineralization and density of the bone. The demand for bone forming minerals during pregnancy, coupled with a low-Ca intake, followed by the decrease in bone density after the menopause, may result in severe health problems for the elderly woman (1).

An elevated intake of cadmium (Cd) through food contaminated by Cd in the soil and drinking water appears to hasten the bone loss that accompanies aging, decreased Ca intake or both of these factors (5). This loss of bone mineral appears enhanced further by numerous pregnancies and lactation periods.

In studies of the relationship between Cd and Ca, investigators used relatively high levels of Cd. Chronic, low-level Cd exposure over an extended period of time has received little attention. Studies have focused on the young growing male or mature female rat with few studies investigating the relationship between Cd and Ca in the adolescent female. To date, no one has reported a study of this relationship in the pregnant adolescent female.

The effect of Cd on bone mineralization, strength and density in the multiparous female rat has not been studied. The present research was undertaken to study Ca and Cd relationships in the albino rat and to determine if an increase in the Ca content of the diet can decrease the rate of bone loss in the multiparous Cd-treated adolescent or mature female rat.

CHAPTER II

REVIEW OF LITERATURE

A. INTRODUCTION

Recent studies suggest the incidence of adolescent pregnancy is increasing and these mothers frequently have several children with short intervals between pregnancies. This segment of the population often consumes diets that contain less than the recommended amounts of Ca and may be at nutritional risk, especially those with marginal Ca intakes.

Cadmium, a heavy metal toxicant, is being used in increasing amounts in a variety of industrial processes. The increasing amounts of Cd polluting the air, water and waste dumps create concern regarding the health of various discrete subgroups in the population, particularly occupational workers and persons already nutritionally stressed. Chronic-low levels of Cd in the diet, coupled with a low dietary intake of Ca, may compromise bone health in the multiparous female.

B. CALCIUM AND THE PREGNANT ADOLESCENT

The number of girls who become pregnant before biological maturity is increasing. Although the fertility rate for women 15-19 years of age has declined, an increase has been noted for girls below 15 years of age. For girls 14 years old, the fertility rate has increased from 6.6 per 1,000 in 1970 to 8.0 per 1,000 in 1980 and this upward trend

is continuing (2, 5). Data for girls 12 and 13 years old are unavailable but pregnancies in this age group appear to be on the increase. In 1980, juvenile pregnancies accounted for 20% of all births. This high percentage may be due partly to the decline in the fertility rate for women over 20 years of age (3, 5).

Pregnancies in girls between the ages of 13 and 16 are a great risk for both the mother and the child. Due to the added stress of growth, girls under the age of 17 have greater nutritional needs than older, biologically mature women. Failure to obtain adequate nutrients during this critical time may compromise growth potential in the girl by depleting the nutrient reserves needed for her growth as well as that of her offspring (6). Pregnancy, whether or not followed by lactation, superimposed on skeletal growth, further increases the need for Ca and other bone forming nutrients.

Balance studies indicate the fetus acquires over one-half of the Ca stored by the mother during gestation which results in a severe drain on Ca stores (1). During normal lactation, the mother loses between 0.15-0.50 g of Ca through the breast milk each day. Even with relatively high dietary Ca intakes, Ca is taken usually from bone reserves during this time. Closely spaced pregnancies and lactation periods may result in appreciable Ca depletion of the mother. This may occur even with apparently adequate Ca intakes because the mother has little opportunity to replete Ca stores between pregnancies (1).

Women, especially teenagers, usually have suboptimal intakes of Ca (5). Results of the first Health and Nutrition Examination Survey

indicate that, at all income levels, a substantial portion of adolescent girls have less than the recommended Ca intake (1, 7).

C. CADMIUM

Sources of Environmental Cadmium

Cd is a heavy metal that has been found to be toxic to man and animals. It is present in the air, soil and water in most parts of the world but the highest concentrations are around industrial plants and metal smelters (4). With the increased use of coal for energy production, areas surrounding coal-fired electricity generators are becoming heavily contaminated with Cd (8).

Pollution of the environment with Cd dust may result from the smelting and refining of zinc (Zn) since one of the industrial sources of Cd is a by-product of Zn production. Cd is volatile at temperatures used in Zn processing so Cd dust is released from the smokestacks of Zn refineries. This dust settles around these industrial facilities contaminating water, soil and plants (4, 8). The Cd concentration in soil ranges from 0.5 to 1.0 mg per kg. However, concentrations may be above 20 mg per kg in mining areas. Concentrations above 2.4 mg per kg in agricultural soil are considered very high (9).

Soil and water may be contaminated with Cd through the extensive use of superphosphate fertilizers, pesticides and other agricultural products that are contaminated with the metal. Cd concentrations of superphosphate fertilizer may exceed 8 mg per kg. At this level, Cd concentration in the top soil is increased (9). Cd from

these sources, once incorporated into the soil, may increase the concentration of Cd in the water and crops (10).

Cd can enter the food chain of man through a variety of sources. Polluted water frequently is used for drinking or irrigation of crops. Foods, such as rice, which require flooding the fields, can be polluted heavily with Cd through contaminated irrigation water. This could increase the Cd in the top 20 cm of soil by as much as 6% (9). Animals fed contaminated water and plants, fish caught from polluted streams and contaminated plants contribute to human Cd consumption. Foods and beverages also can be contaminated with Cd through the use of glazed pottery or enameled steel containers (4).

As early as 1942, impaired health of alkaline accumulator factory workers was associated with occupational exposure to Cd (11). A high-Cd intake is an occupational hazard for persons employed in factories manufacturing plastics, paints, batteries and electroplated products since these items have Cd as a component. Cd used in these industries may be inhaled or absorbed through the skin as well as through the gastrointestinal tract (4, 8, 10).

Daily Intake and Absorption of Cadmium

The daily intake of Cd by humans has been studied in various countries such as Sweden, Japan and the United States. Recently, the body burden of Cd has increased in industrialized countries. The daily intake of Cd by people who smoke is double that of nonsmokers (11). The daily Cd intake from all sources varies from 25 to 75 µg with the average about 50 µg. However, based on recent studies in

Sweden and Belgium, estimates have been reduced (11). When the amount in the drinking water exceeds 5 ng per g the daily intake of Cd is increased by about 10 μg. Because approximately 90-95% of the Cd taken into the body is excreted through the feces as unabsorbed Cd, the daily apparent retention ranges from 2.5 to 7.5 μg. Although a large percentage of an ingested dose of Cd is excreted rapidly in the feces, the absorbed Cd is eliminated very slowly through the urine, with a resulting biological half-life of 10 to 30 years. Due to this long half-life, chronic exposure to the metal leads to considerable accumulation (4).

The two main routes of Cd absorption are through the lungs and gastrointestinal tract but soluble Cd compounds may pass through the skin. Gastrointestinal absorption has been studied by a variety of methods including acute exposure of animals to radioactive Cd or as chronic exposure by adding Cd to the drinking water or food of animals. Regardless of the methods used, investigators have found a long term retention rate of approximately 1% in all of the species studied. When long term exposure is affected through the addition of Cd to the drinking water, the Cd accumulation in the liver and kidney appears to be approximately proportional to the intake (4).

Influence of Dietary Calcium Intake On Cadmium Absorption

The amount of Cd absorbed through the gastrointestinal tract is influenced by a number of nutritional factors. Animal studies have shown that low-Ca and protein intakes increased the absorption of Cd

by the intestine. Studies involving humans have demonstrated that subjects with low-iron (Fe) stores absorb more Cd than those with adequate stores. This suggests females, who have marginal-Fe stores, may have a greater absorption than males (11). Larsson and Piscator (12) found increased Cd accumulation in liver and kidney of female rats fed a low-Ca diet during chronic exposure to Cd when compared to levels in rats fed a diet adequate in Ca. Washko and Cousins (13) also found greater accumulation of radioactive Cd in various tissues of male rats fed a low-Ca diet for four weeks prior to Cd dosing as compared to controls. Significantly greater amounts of radioactive Cd were found in intestinal mucosa, liver, kidney and other organs; lower levels were found in the feces of rats fed low-Ca diets than in rats fed a normal-Ca diet. Because there was no difference in mean body weights of the two groups of rats, the increase in body Cd uptake appeared attributable to an increase in intestinal absorption.

Further evidence for the increased accumulation of Cd in Ca-deficient animals is furnished by Kobayoshi (14) who fed mice a low-Ca, Cd-polluted rice diet. He found liver and kidney Cd concentrations 300-400% higher than those in mice fed a similar diet but adequate in Ca.

It has been well established that chronic exposure to high levels of Cd suppresses food intake; therefore, body weight is decreased. Using an equalized feeding regimen, Takashima, Nishino and Itokawa (15) found a higher incidence of liver, kidney and bone abnormalities in the Cd-treated rats when compared to control animals. Male rats

weighing approximately 120 g were fed a control diet sufficient in Ca or the control diet plus 50 ppm cadmium chloride ($CdCl_2$) or a Ca-deficient diet containing 50 ppm $CdCl_2$ for 45 days. Animals in the first two treatment groups were fed the amount of feed eaten by the third group to equalize the food intake. Cd concentrations in liver and kidney were higher in the Ca-deficient Cd-treated rats than in those fed a diet containing sufficient Ca plus Cd. Histological studies revealed that the thinning of cortical bone was greater in the Ca-deficient Cd-treated rats than it was in the control rats. Thinning of the bone cortex also was observed in the Ca-sufficient Cd -reated rats but to a lesser extent than in rats fed the Ca-deficient diet containing Cd.

Degenerative changes in the liver and kidney were more prominent in the rats fed the Ca-deficient Cd diets than in rats fed the Ca-sufficient Cd diets. Results of this study suggest these abnormalities are related to Cd toxicity and low-Ca intake and not to decreased food consumption.

Manifestations of Cadmium Toxicity

In the late 1940s, bone pain experienced by Japanese women in the Zinzu River area of Toyama prefecture was thought to be related to a chronic intake of Cd (16). These women lived downstream from a mine that heavily polluted the river with Cd. Since the river was used as the source of water for cooking, drinking and irrigating the rice paddies, the daily intake of Cd was high. In 1966, the daily intake of Cd by villagers living downstream from the mine was

estimated to be 600 μg per day or 10 times the average Cd level of the Japanese diet (4).

Clinical manifestations of Cd toxicity include bone pain when pressure is applied to the femur, spine or ribs. This condition has been called itai-itai or "ouch-ouch" disease in Japan due to pain when pressure is applied to the bone. There is also a characteristic duck-like gait and a shortening of the stature which may be caused by skeletal deformation. These symptoms often are present for several years before a mild trauma breaks a number of bones causing the person to be confined to bed. In advanced stages of this condition any mild exertion such as coughing may cause the rib bones to break (4, 16).

Extreme loss of bone mineral was found almost always in post-menopausal women who had several children (usually more than 6) and had a relatively high intake of Cd. These bone changes also occurred in multiparous but not nulliparous women or men, perhaps due to the heavy demand for Ca during pregnancy and lactation (4, 17, 18).

In studies prior to 1955, investigators reported severe decalcification of bone in factory workers exposed to Cd dust and fumes. However, decalcification was not present in all exposed persons so this symptom was not given much attention at that time. It should be noted that the majority of individuals who have considerable bone demineralization following Cd exposure are from nations such as Japan and France where there is not a high consumption of Ca and vitamin D. These bone changes have not been seen in individuals

from countries where traditionally there is a high dietary intake of Ca and vitamin D (4).

Effect of Cadmium Intake on Kidney Function

Cd ingestion has been known to affect the lungs, liver and kidneys as well as the bone. The effects of Cd on lung and liver tissues have not been studied extensively but increases in serum gamma globulin and pulmonary edema have been reported after acute Cd poisoning (19).

Prolonged exposure to Cd dust results in kidney damage. Proteinuria is seen usually in these Cd-exposed workers but frequently ceases when exposure to Cd is stopped. Cd-exposed workers have a higher incidence of kidney stones than do other workers. These stones are mainly calcium-phosphate stones which suggests a disturbed Ca metabolism (4).

Renal changes occurring after long term occupational exposure to Cd usually are not observed until 10-15 years later. Cadmium concentration in liver and kidney of Cd smelter workers was measured by neutron activation to correlate tissue burden, duration of exposure and organ dysfunction. Proteinuria was present in workers exposed to Cd for more than 20 years but it was observed rarely in workers with less than 10 years of exposure to Cd. Liver Cd concentrations increased with increasing length of exposure (20).

Nephrotic lesions have been found in Cd-dosed mice. Changes in the composition of the proximal tubule cells resulted in changes in membrane permeability. A rise in sulfur (S) levels in the distal tubule

mitochondria and cytoplasm agrees with reports of a S-rich metallothioien which can bind Cd in the distal and collecting tubules in rats. In an attempt to clarify the effect of Cd on renal function, Itokawa and co-workers (21) fed male rats diets either adequate or deficient in Ca and drinking water with or without 50 ppm of $CdCl_2$ for 120 days. At the completion of the treatment period, tissue and blood analyses were performed. Hematocrit levels were decreased, serum phosphorus (P) levels increased and serum-Ca levels decreased in all the Cd-treated rats regardless of dietary Ca level. Kidney tissue appeared whitish yellow and hypertrophied and the relative kidney weight was increased in the Cd-treated groups. Microscopic examination of these kidneys revealed tubule and glomeruli degeneration. Dietary Ca exhibited little effect on the parameters measured.

In order to determine the extent and nature of renal damage in chronic Cd poisoning, Itokawa and colleagues (22) fed female rats diets either adequate (0.6% of diet) or low (0.1% of diet) in Ca. One-half of the animals in each treatment group were exposed to 100 ppm Cd in the drinking water. At the end of 60 days decreased hematocrit and increased blood urea nitrogen and serum P levels were noted in all the Cd-treated rats. Blood urea nitrogen levels were higher in the low-Ca Cd-treated rats than in the adequate-Ca Cd-treated rats. Serum Ca was unchanged by treatment but serum P in the Cd-treated rats was 1.7 mg per dl which was higher than the level in the non-Cd-treated rats.

Renal function tests indicated inulin and P clearance decreased significantly in all the rats administered Cd. These tests also

indicated that urinary Ca excretion in Cd-treated rats was greater than in the normal rat regardless of Ca treatment.

Histological analysis of the kidney of the rats treated with Cd indicated degenerative alterations in the cells of the proximal tubules. The damage was more prominent in the rats fed the Ca-deficient diet than it was in rats fed a Ca-sufficient diet at the same level of Cd administration. Analysis of kidney tissue showed higher levels of Cd in the rats fed the Ca-deficient diet than in rats fed the Ca-sufficient diet.

Washko and Cousins (23) fed male rats either a normal (0.6%) or low-Ca (0.1%) diet with or without 25 ppm Cd in the drinking water for an 8 week period. Mineral analysis of liver and kidney indicated Cd present only in the organs of the animals which had received Cd in the drinking water. Rats fed the low-Ca diet had higher Cd levels than did the rats fed the normal-Ca diet.

Effect of Cadmium Intake on Bone Calcium

There is conflict in the literature concerning the mechanism by which Cd affects bone. Some investigators believe Cd has no direct effect on bone and that decreased bone mineral content in Cd exposed animals is due to its effect on mineral metabolism and kidney function. However, others have reported that bone changes occur before any indication of renal damage is evident.

Kawai and co-workers (24) found atrophy of trabecular and cortical bone in male rats fed various levels of Cd in the drinking water. Since bone lesions were found in rats consuming water with 10 ppm Cd

while renal lesions were not noted at this level of Cd, the authors suggested bone is susceptible more to low doses of Cd than is the kidney.

The long term effect of Cd on bone mineralization was studied by Piscator and Larsson (25). Diets containing 0.04% or 1.0% Ca were fed to female rats for 13 months. Cd was administered in the drinking water at either 0, 2.5, 5.0 or 10.0 ppm for the first 7 months and then at one-half of these levels for the remaining 6 months. Ca^{45} was injected and the animals were sacrificed 72 hours later. Cd concentration was determined in liver and kidney and Ca^{45} activity and ash were determined in bone. A significant decrease occurred in the bone mineral content of rats fed the Ca-deficient diet and exposed to either 5 or 10 ppm Cd. There was increased Cd retention in the liver and kidneys of Ca-deficient rats exposed to the 5 and 10 ppm which indicated Ca deficiency resulted in greater absorption of Cd. The tibia of animals fed a low-Ca diet and receiving the largest amount of Cd had the highest rate of Ca^{45} uptake of all treatment groups.

Work by Washko and Cousins (23) demonstrated that the bone of male rats fed a low-Ca diet (0.1%) contained less ash than did those of rats fed a normal-Ca diet (0.6%). The bone ash was decreased further by chronic exposure to 25 ppm Cd in the drinking water. Results also indicated that bones of rats fed a low-Ca diet contained higher levels of Cd than did those of rats maintained on diets containing normal-Ca levels.

Ando and co-workers (26) used radioactive Ca to determine the fate of this mineral in animals continuously exposed to Cd. Ten-week

old female rats were pretreated with an intravenous injection of physiological saline solution containing radioactive Ca. Fifty days later, the animals were divided into two groups with one group dosed with Cd daily for 6 months. Radioactive Ca in urine and feces was measured every 20 days. Significantly greater amounts of radioactive Ca were found in the feces of rats dosed with Cd than in the feces of control rats. These findings indicate Ca resorption from bone increased in Cd-treated rats and that this Ca was excreted through the gastrointestinal tract.

Yoshiki and co-workers (27) fed weanling male rats diets containing 0.45% Ca and 0.3% P with the levels of Cd ranging from 0 to 300 ppm. Bone growth was retarded in animals fed 100 ppm Cd and above with the degree of retardation proportional to the Cd intake. A roentgenogram indicated the bone abnormalities present were due to an inhibition of bone formation rather than an acceleration of bone resorption. These findings suggest Cd poisoning results in osteoporotic rather than osteomalacic changes in the bone of the growing rat.

Many investigators have found bone demineralization following chronic Cd exposure in rats fed a low-Ca diet. This has been attributed to decreased absorption of Ca by the gastrointestinal tract, to increased bone resorption for maintenance of blood Ca or to impaired renal function which results in increased losses of Ca through the urine. Although many studies have been conducted to determine the effect of Cd on dietary Ca utilization the results remain controversial.

Sex Related Differences in Cadmium Retention

Epidemological studies indicate only women, especially those who have a low intake of Ca and vitamin D, suffer the bone lesions that result from chronic Cd intake. A number of studies have been undertaken to determine the relationship of sex to the absorption and retention of Cd.

In a study of Cd concentration in human Japanese cadavers, Sumino and colleagues (28) found a higher concentration of this metal in females than in males. The average Cd concentration in the liver was 36 μg/g for males and 58 μg/g for females. The same tendency held for the concentration in the kidney since it was 3.2 μg/g for males and 8.1 μg/g for females. When Cd concentration in organs of Japanese and Swedish cadavers was measured, females had higher concentrations than did males regardless of nationality (29).

Iwao and co-workers (30) measured Cd in the organs of 394 Japanese cadavers. Sampling was designed to include males and females in all age ranges and from several geographic areas of Japan. Results indicated that the organ burden of Cd increased with increasing age up to age 30 then leveled off. Liver and kidney Cd levels in women exceeded those of men. A difference in body size could not account for the difference in amount of Cd in the organs since the livers of females contained twice the amount found in the livers of the males.

Kello and co-workers (31) used ^{115m}Cd to study the relationship of sex and dietary Ca intake to Cd retention. Male rats retained the

lowest amount of an oral dose of ^{115m}Cd regardless of dietary level of Ca while females retained more Cd whether ovarectomized or not and regardless of the dietary Ca level. This suggested the presence of the male hormones may reduce Cd retention. Regardless of sex, the rats fed a diet containing a low level of Ca retained a larger dose of ^{115m}Cd than did the other groups and the rats fed a high-Ca diet retained the least amount of ^{115m}Cd.

In a study involving male, female and castrated rats fed a low-Ca, low-vitamin D diet and treated with Cd, Yoshikawa (32) found both males and females developed osteoporosis but it was more pronounced in the females. The bones of the female rats exhibited decreased Ca and P content while no effects were seen in the male or castrated rats.

Cadmium and Its Effect on Pregnancy

Since Cd intake has been shown to have a profound effect on females, studies have been conducted to determine the effect of Cd administration on the pregnant female. Sugawara and Sugawara (33) administered 0, 10 or 50 ppm Cd to female rats fed diets adequate in Ca then mated some of them with normal males. Some rats were mated a second time so the effect of Cd on two pregnancies could be measured. They found disturbed Ca and P metabolism in the pregnant rats as compared to nonpregnant animals. There was a decreased accumulation of Ca and P in bones of rats that had been pregnant twice when compared to rats that had only been pregnant once.

In a subsequent study (34), these authors reported a significant decrease in Ca and P in bone of multiparous rats as compared to nulliparous rats. Pregnant and nonpregnant rats were exposed to either 0, 10 or 50 ppm Cd daily in their drinking water for 41 weeks. Serum and femur Ca, P and Cd were measured. Serum Ca and P levels were not affected by parity or Cd treatment. However, multiparous rats receiving 50 ppm Cd had significantly less Ca and P in the femur than did either the nonpregnant rats or multiparous rats treated with 0 or 10 ppm Cd. At a Cd level of 50 ppm in the drinking water, the multiparous rats accumulated twice the quantity of Cd in bone as either nulliparous or multiparous rats treated with 0 or 10 ppm Cd. Bone of multiparous rats treated with 50 ppm Cd contained seven times as much Cd as bone of rats treated with less Cd.

Pond and Walker (35) investigated the effect of Cd and dietary Ca on the reproductive performance and tissue mineral concentration in the female rat and her offspring. Mature female rats were mated with fertile males and then assigned to one of four treatment groups: low Ca (0.1%) low-Cd (0 ppm), low-Ca high-Cd (200 ppm), high-Ca (0.96%) low-Cd, or high-Ca high-Cd. The addition of Cd to the diet decreased feed consumption. At parturition the dams were sacrificed and the tissues prepared for analysis. Tissue analyses of liver and kidney indicated Cd concentration was greater in rats fed a low-Ca diet than in those fed a high-Ca diet. This may be due to the protective effect of high-dietary Ca on reducing gastrointestinal absorption of Cd. Although not significant, there was a trend toward decreased

litter size in dams fed high-Ca high-Cd diets. However, the average pup birth weight was significantly lower when Cd was included in the diet of the dams.

D. BONE DENSITY AND BONE STRENGTH

Development of Methods to Measure Bone Density

Due to the medical implications of bone loss, considerable research has been devoted to the development of a noninvasive technique to measure loss of bone mineral. The discovery of X-rays led to the ability to determine bone loss both qualitatively and quantitatively. Absorption of x-rays by bone has been compared to the absorption of x-rays by various standard materials since the 1940s. Meema and Meema (36) used a potassium hydrogen phosphate solution as the absorption medium but a solid such as 6061 aluminum (Al) alloy (37) was used more commonly. Measurement of bone density using roentgenograms was refined in the 1950s and 1960s. During the 1950s, Mack and her colleagues (38) developed a method for quantitative measurement of bone mineral mass which was used to determine the density of bone in living subjects. This refinement of the earlier instrument of Brown and Birtley improved the reproducibility of the technique, aided data collection and simplified calculations. The complicating effects of differences in bone size, in amount of soft tissue surrounding the bone and in bone matrix were minimized with this technique. The use of this method enabled investigators to measure small amounts of bone loss not determined readily by roentgenograms.

Following the work of Mack and Brown and Birtley, an X-ray densitometer was developed by researchers at The University of Tennessee, Knoxville, for measurement of the bone density of the left phalanx 5-2. This instrument used a collimated X-ray beam to reduce scatter. The source of energy was a low intensity X-ray beam with a scintillation detector joined to a photomultiplier tube eliminating the use of x-ray film. A linear absorption curve was recorded on graph paper by an X-Y recorder (39) incorporating a modification of the pen and ink recorder previously developed by Brown and Birtley (40). This recorder traced x-ray exposure of the stepped wedge and of the bone that was to be evaluated. A standard alloy wedge of 92.8% Al and 7.2% Zn which is closely equivalent in absorptive capacity to bone mineral was used. The bone in the left little finger of the test subject is compared to the standard wedge to derive a bone-density index. The use of this technique allows bones of different densities to be compared but is not to be used as a measure of absolute density. This index is expressed as X-ray equivalent g of alloy per cubic cm of bone. Careful technique reduces the error that is a problem with this method.

Various other methods of measuring bone density have been used but the subjects must be either hospitalized or confined. Although not suitable for diagnosis, densitometry using roentgenograms, remains the best in vivo technique for the study of bone mineral in groups of human subjects (1).

Selected Factors That Influence Bone Density

Bone in the skeleton varies in hydroxyapatite content and the density depends to a great extent upon the mechanical stress placed on the bone. Load-bearing bones contain a larger amount of bone mineral than non-load-bearing bones and therefore, have a greater bone density. When skeletal bones are compared, the femur contains a medium amount of hydroxyapatite. Shraer and co-workers (41) found the density of bone also varies along the length regardless of the kind of bone. The distal portion of the bone has the lowest density, the midsection has medium density and the proximal section the highest density. Since the average density of the three sections is very close to that measured for the midshaft, this section of bone is usually measured and its density is used as a measure of the density of the bone.

Cortical bone mass is a derived calculation so it is not a biological constant as is cortical bone density. Cortical bone density varies from site to site, has individual variation and is somewhat dependent on the body build of the subject. Stocky subjects tend to have greater bone density than their lean counterparts. These calculations may be used in studies in which bone density is measured in various groups of people or animals but is not to be used for diagnostic purposes (1).

Determination of Bone Strength

A variety of instruments have been used to measure strength of bone. Nokso-Koivista and colleagues (43) found good correlation among bone strength, bone density and bone ash in human cadavers.

Both normal and osteoporotic bone breaking load was measured using an instrument constructed in their laboratory. Their instrument, similar to one designed by Stucki (44), consisted of a special measuring pin which pressed on the specimen. The force required to break the bone was taken as the strength of the bone. Reproducibility of results was found to be acceptable.

A hydraulic tensile testing instrument was used by Engesaeter (45) to test bone breaking strength. With this instrument, the force is increased at a constant rate with the maximum load applied taken as the strength of the bone.

Universal testing machines were used by both Okonkwo (46) and Ogoski and associates (47) in their studies of bone strength. These machines employ a load cell, in a compression mode, attached to a moving crosshead. The load cell is used to measure the force required to break the specimen. As with other tests, the maximum load applied is considered the strength of the bone.

As early as 1880, engineering techniques were applied to the study of bone when Messerer made extensive tests on the strength of intact bone. The breaking stress of a material is expressed in terms of the load per unit area which the material supports up to the rupture point. The cross-sectional area must be known to calculate the breaking stress; therefore, most investigators measure breaking load which may be used as a strength index since it only entails measuring the greatest load applied (42). Breaking load is the greatest amount of force withstood by the bone before breaking (48).

Several approaches have been taken to measure the strength of bone. In species such as human and bovine, sections of bone have been used due to the size of intact bone. The use of small animals such as the rat and guinea pig have enabled strength measurements to be made on the entire bone (48, 49).

Variability in the size and shape of bone causes many problems in strength of materials tests. The testing of each type of bone presents its own unique set of problems. The problem of cross section variability can be reduced when large bones are used since uniform sections can be cut from the bone and machined to a specific size and shape. However, if several sections of bone are cut from a single specimen, variability may still be a factor. Mechanical properties differ according to the site from which the bone section is taken as well as from bone to bone. The placement of the bone in a testing instrument also plays a role in reproducibility of results; therefore, care in placement of the bone in the instrument is essential for consistent measurements (48, 49).

It is not possible to machine bones of small animals to a uniform size so they must be tested intact. Testing of intact bone usually is more difficult than sections of large bones since intact bone usually is not perfectly straight and is difficult to position in the holders of any instrument used to break the bone. The cross-sectional area and shape vary along the entire length of the bone with intact bone containing both cortical and spongy bone (48, 49).

The assumptions frequently made that the cross-sectional area is a hollow ellipse and the cortex is of uniform thickness are not valid.

However, these assumptions are made usually so that breaking stress of bones in animals given various experimental treatments may be compared to provide relative values (48, 49).

Bone Strength in Relation to Dietary Calcium

Among the first to study bone strength in relation to Ca intake was Bell and his associates in 1941 (50). Apparatus for bending, twisting, deflection and breaking tests were devised and standard formulae for strength of materials were used to calculate bone strength. Weanling male rats were fed several diets ranging from 0.075 to 1.39% Ca for eight weeks. The animals then were sacrificed and strength of the femur was determined. They found an increase in the strength of the bone as the quantity of Ca in the diet increased but the strength began to plateau when the Ca content reached 0.4% of the diet. Bones used in the strength tests were air dried with the length of time of exposure to air before testing of little to no consequence in the results of the test.

Rowland and his colleagues (51) used a modified Allo-Kramer shear press to test the strength of chick tibia. One of nine experimental diets varying in Ca and P content were fed to one day old chicks for 28 days. The Allo-Kramer shear press was used to measure the force required to break the tibia. They found increasing breaking load with increasing Ca content of the diet and obtained good correlation between breaking load and ash content of the tibia. They suggested breaking load could be used to assess adequacy of mineral intake instead of quantity of ash in studies involving Ca and P.

In a study of the effect of dietary Ca on breaking load of the rat femur, Salomon and Volpin (52) found the level of Ca in the diet did not affect the bone length but the diameter and weight of bone were affected adversely by a decrease in the Ca level of the diet. Female weanling rats were fed a low-Ca (0.02-0.03%) diet or a control (0.43%) diet. Animals were sacrificed at 7, 14 or 28 day intervals and breaking load of the femur was measured using an apparatus of their own design. They observed a decrease, with time, in the strength of the bone in rats fed the low-Ca diet when compared to those animals fed the normal-Ca diet. Riggins and co-workers (53) also found Ca restriction significantly decreased density, diameter, cortical thickness and strength of the bone.

E. SUMMARY

The relationship between dietary level of Ca in animals exposed to Cd in terms of bone mineralization is still unclear. Evidence suggests that a chronic intake of Cd, especially when accompanied by a low dietary intake of Ca, is associated with low mineral content of the bone. The cause of this is controversial. Some investigators propose the low bone mineral content in Cd toxicity is due to the malabsorption of Ca and other bone forming nutrients from the gastrointestinal tract. Others attribute the low bone mineral content to excess Ca excretion due to the Cd damaged kidney. Still other investigators believe Cd has a direct effect on bone causing demineralization. Many researchers suggest the cause of low bone mineral content with Cd intoxication

may be multifactorial and involve malabsorption of Ca, imbalance in the ratio of Ca and P and a direct effect of Cd on the bone.

Only in the last several years has research been conducted to study the relationship among bone mineral, bone density and bone strength. Although the effect of Cd on bone Ca and P has been investigated, no investigators to date have determined the effect of Cd on bone strength and bone density.

Much of the work involving the toxicity of Cd has been done with male rats or young, growing rats. Limited work has been done with females and few studies involve the pregnant animal. No one to date has investigated whether or not increasing the level of Ca in the diet of the female rat will alleviate the toxic effects of Cd on bone during the reproductive life of the animal.

CHAPTER III

EXPERIMENTAL PROCEDURE

A. INTRODUCTION

Weanling female rats were fed diets containing one of three levels of Ca and one of four levels of Cd in the drinking water to determine the effect on the reproductive performance, plasma Ca and P levels and bone mineral content as well as bone-density index and strength. Rats were exposed to males either as adolescent (55 days of age) or mature (110 days of age) animals. One-half of the animals were sacrificed at the completion of the first lactation period and the other half after the completion of four pregnancy and lactation periods. Reproductive performance was assessed, plasma and bone were analyzed for Ca and P and the density and strength of the femur were determined.

B. DIETARY TREATMENT AND CARE OF ANIMALS

One thousand weanling Sprague-Dawley female rats from the stock colony of the Comparative Animal Research Laboratory, Oak Ridge, Tennessee, were used in this study. The rats were housed in groups of four animals in stainless-steel, wire-bottomed, hanging cages. From the time of weaning to the beginning of dietary treatment, all rats consumed rat chow and tap water. At 24 days of age, the rats were divided into three groups and were fed, ad libitum, semipurified diets containing one of three levels (low, normal and high) of Ca (0.3, 0.6, or 0.9% of the diet). All diets contained 0.5% P, the level considered

adequate for the pregnant rat by the National Research Council. The compositions of the diets are found in Table 1.

At 42 days of age, each Ca level group was divided into four subgroups and given one of four levels of Cd (0, 1, 5, or 10 ppm) in the drinking water as $CdCl_2$ (Figure 1). The animals receiving no Cd were given double distilled water (DD H_2O). The Cd and Ca treatments were continued throughout the study in a four by three factorial design.

When the rats reached 55 days of age, one-half of each dietary treatment group was exposed to stock colony males of known fertility (these females constituted the adolescent group, AG). Two males were placed in each cage every evening and removed the following morning for 6 consecutive days. The other half of the animals in each dietary treatment group were not exposed to males until 110 days of age and constituted the mature group (MG).

Approximately two days before the rats were due to litter, the animals were placed in individual plastic littering boxes with stainless-steel tops. The pups were weighed within a few hours after birth. At three days of age, the pups were again weighed and four male and four female pups were chosen at random to remain with each dam to standardize the litter size. If this sex combination was not possible, 8 pups were selected randomly to standardize the litters. In the litters with less than 8 pups, cross fostering from dams in the same treatment group was done to standardize litters. All pups not remaining with their mothers or used for cross fostering were removed from the

Table 1

Composition of the diets

Component	Calcium		
	0.3%	0.6%	0.9%
		g/kg	
Casein, Vitamin-Free[1]	250.0	250.0	250.0
Methionine (DL)	3.0	3.0	3.0
Sucrose	289.8	282.3	274.9
Cornstarch	305.0	305.0	305.0
Vegetable Oil	100.0	100.0	100.0
Vitamin Mix[2]	22.0	22.0	22.0
Ca/P Deficient Salt Mix[1]	13.4	13.4	13.4
Calcium Monophosphate	6.8	6.8	6.8
Potassium Diphosphate	7.5	7.5	7.5
Calcium Carbonate	2.5	10.0	17.5

[1]Teklad, Madison, Wisconsin.

[2]ICN Nutritional Biochemicals, Cleveland, Ohio.

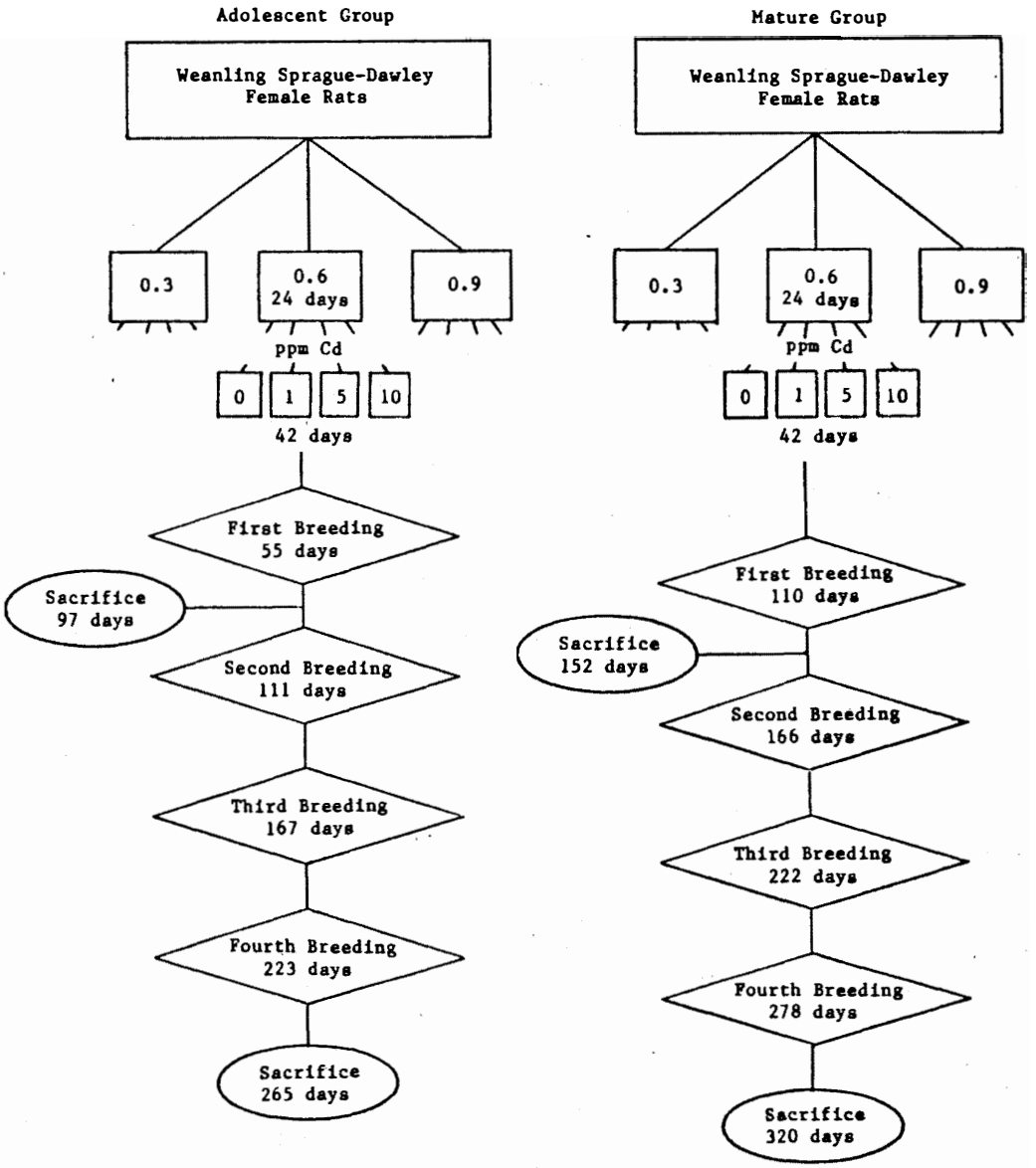

Figure 1. Schematic Diagram of Assignment of Females to Treatment Group from the Beginning of the Study.

study. Animals that did not conceive, had stillborn litters or had less than three pups at three days postpartum were removed from the study.

When the offspring reached 21 days of age they were taken from the dams, weighed and removed from the study. Approximately ten dams per dietary treatment group in each age group (AG and MG) were selected randomly and sacrificed. The remaining dams were housed, by dietary treatment, in groups of three or four in hanging stainless-steel cages. After a two-week recovery period, they were exposed to males as described previously. These dams were allowed to litter and were treated in a manner identical to that used following the first pregnancy. The same cycle of pregnancy, lactation with 8 pups, removal of pups at 21 days and two weeks of rest was repeated for the third and fourth pregnancies. All remaining dams were sacrificed at the completion of the fourth lactation period. At the completion of the study, tissues from dams sacrificed at the end of the first lactation period and those from dams that completed four consecutive lactation periods were analyzed.

At the time of sacrifice, the animals were anesthetized with methoxyfurane (Metofane) and blood was obtained by heart puncture using a 10 ml syringe containing 0.2 ml heparin (U.S. Biochemicals). The blood was placed in acid-washed tubes and centrifuged at 1000 x g for 10 minutes in a refrigerated centrifuge. The plasma was removed with a disposable pipette and placed in individual plastic bags (Whirl-Pak, Nasco, Inc.) and sealed. The plasma was frozen and kept at -4°C until Ca and P determinations were performed.

After sacrifice, the femurs were removed and cleaned of adhering tissue. Wet weights were recorded after which the femurs were allowed to air dry for 48 hours before they were placed in individual Whirl-Pak plastic bags. The femurs were stored at room temperature until bone-density index, strength and mineral content were determined. The right femur was used for strength and density measurements and the left femur used for the determination of Ca and P.

C. ANALYSIS OF PLASMA

Analysis of Plasma Phosphorus

Plasma samples were thawed and plasma inorganic phosphate (P_i) was determined on duplicate aliquots by the method of Dryer (54). After deproteinization with 10% trichloroacetic acid (TCA), the P_i was converted to phosphomolybdate by the addition of ammonium molybdate. N-phenyl-P-phenylenediamine monohydrochloride was added to reduce the phosphomolybdate and produce a blue color. The absorbance (A) of the colored complex was measured photometrically at 770 nm using a Beckman Model B spectrophotometer and the concentration of P_i was calculated. If the results were not in agreement within 5% on duplicate aliquots, the procedure was repeated. The average value obtained for the duplicate P_i determinations was used for the statistical analysis.

Reagents.

1. Trichloroacetic Acid (TCA), 10%:

   100 g of TCA was diluted with DD H_2O to a final volume of 1 l.

2. Phosphorus Stock Standard:

   0.4381 g of KH_2PO_4 was diluted with 10% TCA to a final volume of 100 ml. This solution contained 1 mg P_i per ml.

3. Phosphorus Working Standard:

   100 ml volumes of working standard containing 2, 4, 6, 8, 10 and 12 µg per ml were prepared by diluting the P_i stock standard with an appropriate volume of 10% TCA. One ml of each standard was used per 3.2 ml final volume for each series of determinations.

4. Ammonium Molybdate, 0.008 M:

   0.5 ml H_2SO_4 was added to 2.475 g $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ to dissolve the ammonium molybdate. This was diluted with DD H_2O to a final volume of 250 ml.

5. N-Phenyl-P-Phenylenediamine Monohydrochloride, Sodium Bisulfite:

   Several drops of 70% ethanol were added to 0.5 g of N-phenyl-P-phenylenediamine monohydrochloride and then diluted to 1:1 with 1% (w/v) aqueous solution of sodium bisulfite.

Procedure. Two-tenths ml of each plasma sample were placed in a tapered centrifuge tube and 1.8 ml of 10% TCA added by Repipet. Ten minutes later the samples were centrifuged for 10 minutes at 1000 x g. One ml of the clear supernatant was removed and transferred to 10-ml tubes. Two-tenths ml of ammonium molybdate and 2 ml of N-phenyl-P-phenylenediamine monohydrochloride were added. The

contents of each tube were mixed using a mechanical mixer (Vortex, Jr.) and allowed to stand for 30 minutes to permit color development. Standards containing 0 to 12 μg P_i per 3.2 ml solution were prepared in the same manner as the samples. Absorbance (A) was determined and calculations were performed as follows:

$$\text{Phosphorus (mg/dl plasma)} = \frac{\text{A of Sample} \times \text{conc of STD}}{\text{A of STD}} \times 1000$$

Analysis of Plasma Calcium

Plasma samples were thawed and Ca determined on duplicate aliquots by atomic absorption spectrophotometry (Instrumentation Laboratories Model 751) and the concentration of plasma Ca calculated. Lanthanum chloride ($LaCl_3$) was added, as described by DeLuca (55), to eliminate interference by other cations. If duplicate aliquots were not within 10% agreement, the procedure was repeated. The average value obtained from the two plasma Ca determinations was used for statistical analysis.

Reagents.

1. Lanthanum Chloride, 0.1%:

   2.54 g $LaCl_3 \cdot 6H_2O$ were diluted with DD H_2O to a final volume of 1 l.

2. Calcium Working Standards:

   Calcium Standard (Fisher) 1000 ppm was diluted with 0.1% $LaCl_3$ to yield working standards of 0.5, 1.0, 1.5, 2.0 and 3.0 ppm Ca.

Procedure. Two-tenths ml of each plasma sample were placed in acid-washed tapered centrifuge tubes and 3.8 ml of 0.1% $LaCl_3$ were added. Ten minutes later the samples were centrifuged for 10 minutes at 1000 x g. The supernatant was decanted into 5-ml tubes and the Ca determined using atomic absorption spectrophotometry at a wave length of 422.7 nm. A lean air-acetylene flame was used with a single element Ca lamp. The instrument was calibrated using the conditions suggested in the operating manual (56). The band width was set at one, the energy level fluctuated between 0.2-0.8 and the background correction was on. The aspiration rate was regulated at 4 ml per 30 second. The 1.0 ppm standard had an absorbance of 92 ± 3, the value suggested by the instrument manual. The absorbance of the other standards indicated they were in the linear portion of the curve. A standard curve using the 5 previously described working standards was entered into the microcomputer of the instrument so that the results were calculated as ppm. The integration time was set at 8 per second.

Calculations mg/dl were performed as follows:

$$\text{Calcium (mg/dl plasma)} = \frac{\text{ppm Ca x dilution factor}}{10{,}000}$$

D. ANALYSIS OF BONE

The length of the left femur of each dam was measured using stainless-steel vernier calipers. After measurements were completed, each femur was weighed on a Sartorius analytical balance and placed in

a labeled 5-ml glass tube. These femurs were dried in an 80°C oven for 50 hours then placed in desiccators. Femurs were weighted to determine dry weight then finely ground using a Wiley mill. A portion of the resulting ground bone was wet ashed with nitric acid (HNO_3) and 30% hydrogen peroxide (H_2O_2) following the suggestions of Gorsuch (57). Ca and P were determined using the reagents and instruments described previously for plasma Ca and P.

Procedure

Approximately 0.100 g of dried ground bone was weighed into tared 125-ml Philips beakers and the weights recorded. Fifteen ml of concentrated HNO_3 were added to each beaker by Repipet. The beakers were covered with watch glasses and the mixture was allowed to digest overnight.

Solutions containing the dissolved bone were placed on a hot plate and boiled vigorously until approximately 2 ml of liquid remained in the beaker. Beakers were removed from the hot plate and allowed to cool. Two ml of 30% H_2O_2 were added to each beaker which was then returned to the hot plate set at medium heat. The solutions were boiled again until approximately 2 ml of liquid remained.

After cooling, the samples were diluted to 20 ml by weight with 0.5% HNO_3 using an Arbor digital balance. The resulting solutions were diluted further 1 to 50 with 0.5% HNO_3 for Ca and P determinations.

Analysis of Bone Phosphorus

Bone P_i was determined by adding one-half ml of DD H_2O to one-half ml of the sample diluted previously. The resulting 1 ml of solution was analyzed for P_i using the same procedure as described for the determination of P_i in plasma. Calculations were performed as follows:

$$\text{Phosphorus (mg/100 mg dry bone)} = \frac{\text{A of Sample x conc of STD}}{\text{A of STD}} \times 1000 \times \text{dilution factor x wt of bone sample}$$

Analysis of Bone Calcium

Bone Ca was determined by atomic absorption spectrophotometry on the diluted sample using the same instrumental parameters described previously for plasma Ca. A solution of 0.5% lanthanum oxide (La_2O_3) was used to dilute the samples to eliminate interference by other cations.

Reagents.

1. Lanthanum Oxide, 5%:

   250 ml concentrated hydrochloric acid (HCl) were added slowly to 58.6 g La_2O_3 to dissolve the La_2O_3. After cooling, the solution was diluted with DD H_2O to make 1 l.

2. Lanthanum Oxide, 0.5%:

   100 ml of the 5% solution of La_2O_3 were diluted with DD H_2O to make 1 l.

3. Calcium Stock Standards:

   prepared as described previously except diluted with 0.5% La_2O_3.

Procedure. A 1 to 20 dilution was prepared using the previously diluted sample of wet ashed bone. The instrumental parameters and standards were the same as outlined previously for determination of Ca in plasma. The absorbances of the standards were determined with the instrument and the standard curve was calculated by the microcomputer of the atomic absorption instrument as described for plasma Ca. A solution of 0.5% HCl was aspirated after 10 samples were analyzed to clear the aspiration mechanism.

Calculations were performed as follows:

$$\text{Calcium (mg/100 mg dry bone} = \frac{\text{ppm Ca} \times \text{Dilution Factor}}{10{,}000} \times \text{wt of bone sample}$$

E. MEASUREMENT OF BONE-DENSITY INDEX

The bone-density index was measured using a Spin-Lab X-ray Bone Densitometer. A collimated X-ray beam was passed through the midpoint of the femur to a photomultiplier tube. The output of this tube was recorded, using a pen and ink recorder, as the X-ray beam was swept laterally across the bone. A tracing was made also of a standard Al alloy wedge. The bone-density index was calculated.

Procedure

The Spin-Lab X-ray Bone Densitometer was calibrated according to the instruction manual (58). A metal stepped wedge of leaves of metal was used to interrupt the X-ray beam and calibrate the pen and ink recorder. The recorder was calibrated after each specimen was analyzed.

The length of the right femur was measured using calipers and the midpoint marked with a fine felt-tipped pen. The femur was placed in the stand with the knee joint down and the ball joint to the left and toward the front of the instrument (Figure 2). The midpoint of the femur was aligned with the path of the X-ray beam. Tracings were made of the X-ray absorption of each specimen as the x-ray swept across the bone.

Calculations for the bone-density index were performed by measuring the area under the curve with a K-E planimeter. Two measurements were made for each curve. If these were not identical, a third measurement was performed and an average calculated. The area under the X-ray absorption curve was divided by the distance the X-ray beam swept across the bone (length of the baseline) to obtain the average constant density. This average absorption was compared to the standard Al wedge tracing to obtain the bone-density index.

F. MEASUREMENT OF BONE STRENGTH

The strength of a material may be determined by measuring the force required to break the material. For convenience the three-point

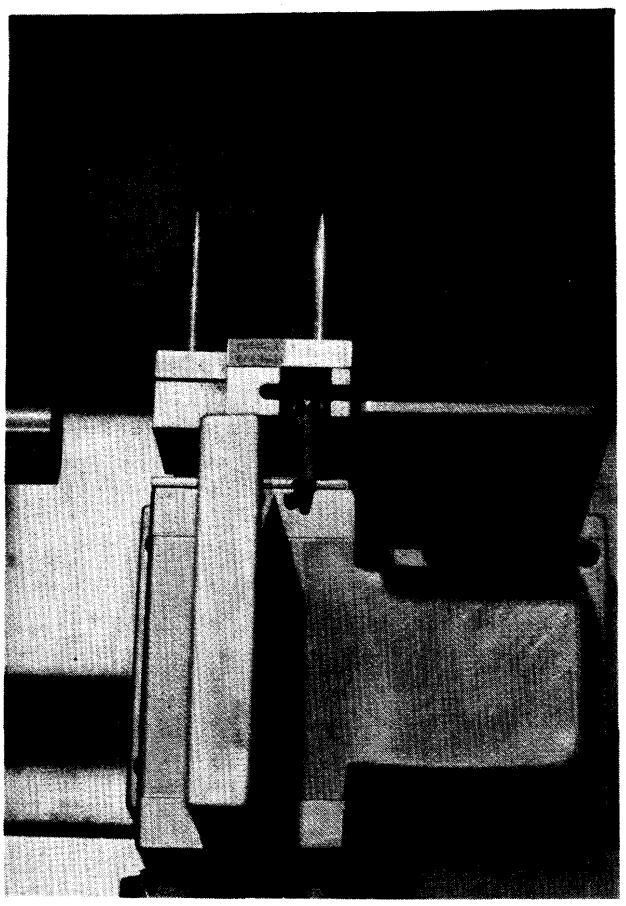

Figure 2. Placement of Femur in Finger Stand of the Bone Densitometer.

bending test was used. Accuracy of the results in three-point bending tests depends upon the homogeneity of the test material. Biological materials such as bone do not always exhibit the degree of homogenity found in nonliving materials. However, since the variability in bone is small, it is probably within the limit of possible error in testing. Therefore, in strength of materials tests, bone material frequently is assumed to be homogeneous. Preliminary testing showed consistency when right and left femurs of the same animal were broken.

Biological materials also pose another problem in strength of materials tests since they frequently are not the same size. Small bones such as the rat femur cannot be machined to make them uniform in size so they must be tested in their original shape. The midpoint of the femorial shaft was used to reduce the experimental error as much as possible. The midpoint of the bone may not be the weakest point but it may be used as a reference point so that bones of animals subjected to a variety of treatments may be compared.

Procedure

The strength of the right femur was measured using a universal testing machine (Instron Corp., Model DD-20). A device, similar to the one described by Stucki (44) was fabricated in the machine shop of the College of Engineering at The University of Tennessee, Knoxville, to hold the bone during testing. The device consists of a support attached to the load cell to hold the bone in position and a moving blade attached to the crosshead (Figure 3).

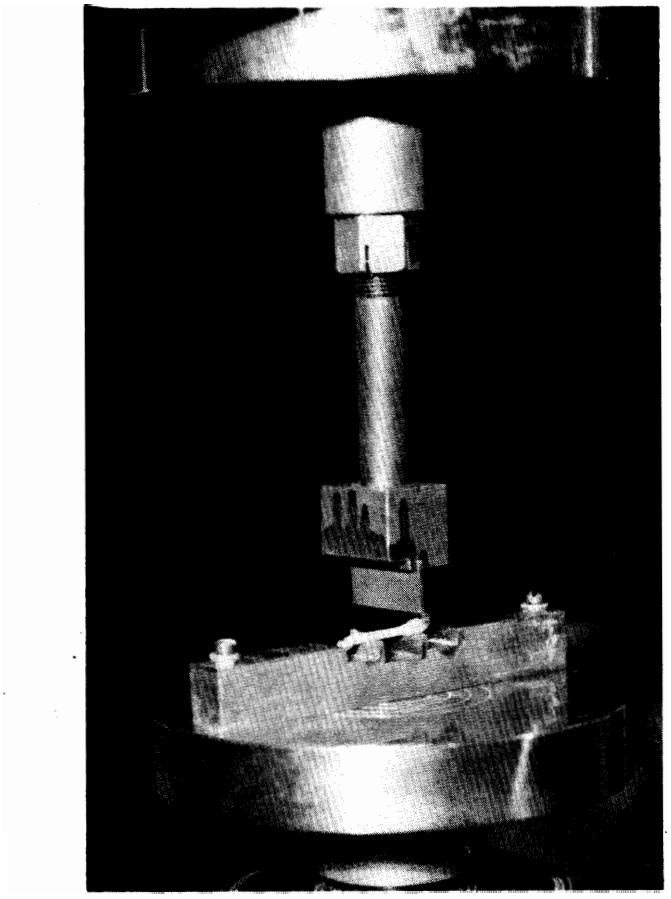

Figure 3. Placement of Femur in Apparatus of the Instron to Hold the Femur During Bone Strength Measurement.

Femurs were positioned for a three-point bending test. The bone rested on two supports and the midpoint was aligned with the blade. Force was applied at the midpoint of the bone with the moving blade attached to the crosshead. The supports and the moving blade were 3-4 mm thick and were not beveled or sharpened. Positioning of the bone in the Instron is shown in Figure 3.

The Instron was calibrated with each inch on the graph paper equivalent to 5 pounds of force. A 50-pound capacity load cell on a range of 0 to 50 was found to give good resolution. Measurement of breaking load was recorded on chart paper as the force required to break the bone. Calculations were performed by measuring the length of the line drawn by the pen on the chart paper when the bone broke.

G. STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was completed using the Statistical Analysis System (SAS) (59) at The University of Tennessee, Knoxville. The experimental design was a four x three x two x two factorial design.

The statistically tested main effects for reproductive performance were Cd, Ca and age. The two-way interactions tested were Cd x Ca, Cd x age and Ca x age. The three-way interaction was Cd x Ca x age.

The statistically tested main effects for plasma Ca and P and bone mineral, density and strength were Cd, Ca, age and parity. The two-way interactions tested in this study were Cd x Ca, Cd x age, Cd x

parity, Ca x age, Ca x parity and age x parity. The three-way interactions were Cd x Ca x age, Cd x Ca x parity, Cd x age x parity and Ca x age x parity and the four-way interaction was Cd x Ca x age x parity.

Procedure GLM (General Linear Model) was used for all ANOVA using the least-squares means of all main effects and interactions because all groups did not contain the same number of animals. When a statistically significant main effect or interaction was noted, PDIFF was used to determine differences between least-squares means.

Significance was tested at the $p < 0.05$ level for the effect of age, parity and dietary treatment on reproductive performance, plasma Ca and P, bone mineral and bone density and strength.

CHAPTER IV

RESULTS AND DISCUSSION

A. REPRODUCTIVE PERFORMANCE

The effect of dietary treatment on reproductive performance may be measured in a variety of ways. Litter size and average birth weight of the offspring are measures of the outcome of pregnancy while weight of the progeny at weaning is a measure, in part, of the lactation ability of the dam as well as reproductive outcome.

Although litter size was not used as a good measure of reproductive performance, the average body weights of viable young were a reflection of the outcome of pregnancy. Many dams had stillborn pups or pups that died shortly after birth.

Dietary Ca had no effect on the birth weight of the offspring at either the first or fourth pregnancy (Table A-1, Appendix). However, Cd treatment at all levels, affected the birth weight of both male and female offspring of both age groups at both the first and fourth pregnancies. The age of the dam affected the birth weight of the female pups after the first pregnancy but the effect was no longer evident for the fourth pregnancy.

The effect of Cd and Ca treatment and age of the dam was evident when the pups were weaned after the first lactation period (Table A-2, Appendix). By the completion of the fourth lactation period, the effect of age had disappeared. There were interactions for Cd and age for both sexes and Cd, Ca and age for males only on

weaning weight after the first pregnancy; these were not present after the fourth pregnancy.

Effect of Cadmium on Birth Weights

Cadmium in the drinking water had a negative effect on the average birth weight of the offspring of the first pregnancy at first breeding regardless of Ca treatment (Table 2). Birth weights for male pups from the first pregnancy ranged from 5.5 to 5.7 g. Male pups from dams given 5 or 10 ppm Cd were significantly smaller than those given 0 or 1 ppm Cd. The weight of female pups from the first pregnancy ranged from 5.2 to 5.4 g. Female pups of dams consuming 5 ppm Cd were significantly smaller than female pups of dams consuming 0 or 1 ppm Cd; however, the female pups of dams consuming 10 ppm Cd were not significantly smaller. Although the levels of Cd used in the present study were much lower than those of Pond and Walker (35), the findings agree with these researchers who observed a significant reduction in birth weight of pups born to dams receiving 100 ppm Cd in the drinking water.

By the completion of the fourth pregnancy, only 10 ppm Cd had an effect on birth weight of the offspring. Birth weight decreased from 6.2 g at 0 Cd to 5.6 g at 10 ppm Cd for the males and from 6.0 g at 0 Cd to 5.5 g at 10 ppm Cd for the females. Pups of dams receiving 0, 1 or 5 ppm Cd showed no difference in weight for either sex but chronic exposure to 10 ppm Cd resulted in offspring significantly smaller than did exposure to other Cd levels.

Table 2

Effect of cadmium and age of dam on the average birth weight of the offspring from the first and fourth pregnancies[1]

Treatment	Number of dams	First pregnancy		Number of dams	Fourth pregnancy	
		Males	Females		Males	Females
ppm		g	g		g	g
			Cadmium			
0	114	5.7 ± 0.1[2a]	5.4 ± 0.1[a]	59	6.2 ± 0.1[a]	6.0 ± 0.1[a]
1	112	5.7 ± 0.1[a]	5.4 ± 0.1[a]	55	6.4 ± 0.1[a]	6.0 ± 0.1[a]
5	100	5.6 ± 0.1[b]	5.2 ± 0.1[b]	44	6.2 ± 0.1[a]	5.8 ± 0.1[a]
10	110	5.5 ± 0.1[b]	5.3 ± 0.1[ab]	54	5.6 ± 0.1[b]	5.5 ± 0.1[b]
			Age of dam			
Adolescent	235	5.6 ± 0.1[c]	5.3 ± 0.1[c]	115	6.1 ± 0.1[c]	5.8 ± 0.1[c]
Mature	201	5.7 ± 0.1[c]	5.4 ± 0.1[d]	97	6.2 ± 0.1[c]	5.9 ± 0.1[c]

[1] Least-Squares Means ± SE.

[2] Means within the same column for each subgroup with different superscripts differ at the $p < 0.05$ level of significance.

Only the female pups of the adolescent dams were significantly smaller than the female pups of the mature dams after the first pregnancy. By the completion of the fourth pregnancy, there were no differences in birth weights of the pups from adolescent or mature dams.

Cadmium had no effect on the number of viable young at either the first or fourth pregnancy. These findings agree with those of Pond and Walker (35) who observed no decrease in litter size after Cd treatment. However, the number of pregnancies appeared to affect the number of viable offspring. For the first pregnancy, litters averaged 9.8 viable pups per dam regardless of the age or dietary treatment of the dams but decreased to 8 viable pups per litter by the fourth pregnancy. This smaller litter size at the fourth pregnancy may have contributed to the trend toward increased birth weight of the pups from the fourth pregnancy. In summary, average pup birth weight increased from first to fourth pregnancy and chronic exposure to 10 ppm Cd decreased average pup weight at birth after four pregnancies and after the first pregnancy in male pups. Birth weight of pups of adolescent dams was depressed by 5 ppm Cd after the first pregnancy but not after the fourth pregnancy.

Effect of Cadmium on the Weaning Weights of Offspring

The weaning weights of offspring from the first pregnancy of dams given 0 or 1 ppm Cd in their drinking water were not significantly different (Table 3). However, at the 5 ppm Cd level a

Table 3

Effect of cadmium, calcium and age of dam on the average weaning weights of the offspring from the first pregnancy[1]

Treatment	Number of dams	Males	Females
		g	g
ppm		Cadmium	
0	114	51.4 ± 0.5[2a]	49.2 ± 0.4[a]
1	112	50.7 ± 0.5[a]	48.7 ± 0.4[a]
5	100	45.9 ± 0.5[b]	44.4 ± 0.5[b]
10	110	38.2 ± 0.5[c]	37.6 ± 0.4[c]
%		Calcium	
0.3	144	45.0 ± 0.4[d]	43.9 ± 0.4[d]
0.6	154	47.1 ± 0.4[e]	45.1 ± 0.4[e]
0.9	138	47.8 ± 0.4[e]	45.9 ± 0.4[e]
		Age of dam	
Adolescent	235	43.8 ± 0.3[f]	42.6 ± 0.3[f]
Mature	201	49.3 ± 0.4[g]	47.4 ± 0.3[g]

[1] Least-Squares Means ± SE.

[2] Means within the same column for each subgroup with different superscripts differ at the $p < 0.05$ level of significance.

significant decrease in average weaning weight of pups occurred and was even more depressed when Cd in the drinking water was increased to 10 ppm. This depression in body weight of the offspring occurred for the first as well as the fourth pregnancy pups (Tables 3 and 4). However, a greater decrease in body weights was noted between the offspring of the dams receiving 5 and 10 ppm Cd after the first pregnancy than after the fourth pregnancy. Mean weaning weight for first litter males of dams given 10 ppm Cd was 13.2 g less than those given water without Cd (Table 3). After the fourth pregnancy the decrease was only 8.6 g for male pups (Table 4). Similar reductions in weaning weights for female pups of dams given 10 ppm Cd were noted also. For the first pregnancy, the female pups of dams given 10 ppm Cd weighed 11.6 g less than the female pups of dams given water with 0 ppm Cd. By the fourth pregnancy the difference was 7.0 g. The difference in weaning weight of pups between the first and fourth litters whose dams were treated with 10 ppm Cd may be due, in part, to the removal from the study of the dams which did not conceive four consecutive times. By the time of the fourth pregnancy, many of the dams receiving 10 ppm Cd had been removed from the study since they either had stillborn litters or had less than three pups remaining three days postpartum. This culling process may have resulted in the elimination of dams less able to adapt to the stress of Cd toxicity. The dams that could produce litters of more than three pups for four pregnancies in spite of Cd treatment were undoubtedly better adaptors to stress and may naturally have had large offspring.

Table 4

Effect of cadmium, calcium and age of dam on the average weaning weights of the offspring from the fourth pregnancy[1]

Treatment	Number of dams	Males	Females
		g	g
ppm		Cadmium	
0	59	54.3 ± 0.6[2a]	51.6 ± 0.6[a]
1	65	54.0 ± 0.6[a]	52.1 ± 0.6[a]
5	44	50.8 ± 0.7[b]	52.1 ± 0.7[b]
10	54	45.7 ± 0.6[c]	44.6 ± 0.7[c]
%		Calcium	
0.3	72	49.8 ± 0.6[d]	43.9 ± 0.4[d]
0.6	75	52.0 ± 0.6[e]	49.9 ± 0.6[e]
0.9	65	51.8 ± 0.6[e]	50.0 ± 0.6[e]
		Age of dam	
Adolescent	115	51.6 ± 0.5[f]	49.5 ± 0.5[f]
Mature	97	51.3 ± 0.5[f]	48.9 ± 0.5[f]

[1] Least-Squares Means ± SE.

[2] Means within the same column for each subgroup with different superscripts differ at the $p < 0.05$ level of significance.

Effect of Dietary Calcium Intake on Weaning Weights of the Offspring

At weaning, there were significant differences in the average body weights of the male and female pups from the first litter depending on the dietary Ca level of the dam as shown in Table 3. Reducing the Ca level from 0.6 to 0.3% of the diet resulted in a 2.1 g decrease in the body weight for males and a 1.2 g decrease in body weight for females. Increasing the Ca from 0.6 to 0.9% of the diet had no significant effect on the average weaning weight of the offspring of either sex. The depressed weight of pups of dams fed low-Ca diets also was evident in the fourth pregnancy especially for female offspring (Table 4). As with the first pregnancy, increasing the dietary Ca from 0.6 to 0.9% of the diet did not increase significantly the weaning weight of the offspring. These results indicate that feeding a low-Ca diet to female rats depressed the growth of their offspring. However, increasing the Ca in the diet above normal levels did not increase the size of the offspring at weaning.

Effect of Age of the Dam on the Weaning Weights of Offspring

Weaning weights of the offspring of the adolescent dams were significantly smaller than those of the mature dams after the first pregnancy as shown in Table 3. Weaning weight was 49.3 g for male pups of the mature dams but was only 43.8 g for male pups of the adolescent dams. There was a similar difference between the average weaning weight of the female pups of the adolescent and the mature dams. This effect disappeared by the completion of the lactation

period following the fourth pregnancy (Table 4). By the fourth pregnancy, the adolescent dams had reached maturity so that their body weight (318 g) was not significantly different from that of the mature dams (338 g) in the study. However, the average body weight of mature dams sacrificed at the completion of the first lactation period was 277 g and the average body weight of the adolescent dams was only 238 g and was statistically different.

Interaction of Cadmium and Age of Dam on Weaning Weights of Offspring of the First Pregnancy

There was a significant interaction between Cd and the age of the dam for the weaning weight of the male and female offspring of the first pregnancy (Table 5). When compared within the sex groupings, both male and female offspring of the mature dams given 0 or 1 ppm Cd weighted significantly more than did any other group at weaning. Weaning weights of the offspring of the adolescent dams treated with 0 or 1 ppm Cd were not significantly different from offspring of mature dams treated with 5 ppm Cd. Weaning weights of the offspring of adolescent dams treated with 5 ppm Cd were not significantly different from the weaning weights of the offspring of the mature dams fed 10 ppm Cd. The adolescent dams fed 10 ppm Cd had offspring which were significantly smaller than any other group. It was apparent that the offspring of the adolescent dams were more affected by Cd ingestion than the offspring of the mature dams.

Table 5

Interaction of cadmium and age of dam on the average weaning weights for offspring of the first pregnancy[1]

Cadmium	Number of dams	Males	Females
ppm		g	g
		Adolescent	
0	65	49.8 ± 0.6[2a]	48.1 ± 0.6[a]
1	61	48.8 ± 0.7[a]	47.2 ± 0.6[a]
5	52	42.8 ± 0.7[b]	41.5 ± 0.6[b]
10	57	34.0 ± 0.7[c]	33.6 ± 0.6[c]
		Mature	
0	49	52.9 ± 0.7[d]	50.4 ± 0.6[d]
1	51	52.6 ± 0.7[d]	50.2 ± 0.6[d]
5	48	49.0 ± 0.7[a]	47.3 ± 0.7[a]
10	53	42.6 ± 0.7[b]	41.7 ± 0.6[b]

[1] Least-Squares Means ± SE.

[2] Means within each column with different superscripts differ at the $p < 0.05$ level of significance.

Interaction of Cadmium, Calcium and Age of Dam on Weaning Weights of Male Offspring of the First Pregnancy

There was significant interaction among the levels of Cd, Ca and the age of the dam on the weaning weight of the male offspring from the first pregnancy (Table 6). Ten ppm Cd in the drinking water decreased the weaning weight of the male offspring of the adolescent and mature dams fed low (0.3%) and normal-Ca (0.6%) diets. Weaning weights of the offspring of the adolescent dams were affected by 10 ppm Cd regardless of the Ca level of the diet. Weaning weights of the male offspring of the adolescent dams fed the low-Ca diet were decreased 14.6 g when the Cd level was increased from 0 to 10 ppm but the weight decrease was only 11.9 g for the male offspring of the mature dams fed the low-Ca diet. Reductions in weaning weights were observed in the offspring of both age groups fed the normal and high-Ca diets and treated with 10 ppm Cd and were lower than the weights observed at the lower Cd levels. The effect of the 10 ppm Cd observed in the male offspring of the mature dams was evident at the 5 ppm Cd treatment level in the adolescent dams.

The mature dams had been treated with Cd twice as long as the adolescents (110 days for the MG and 55 days for the AG) by the completion of the first lactation period. Even though the MG could have built up a greater body burden on Cd than the AG, the offspring of the mature dams were better able to overcome the effect of the Cd than the offspring of the adolescent dams.

Table 6

Interaction of cadmium, calcium and age of dam on average weaning weights for male offspring from the first pregnancy[1]

Cadmium	Number of dams	Calcium 0.3%	Number of dams	Calcium 0.6%	Number of dams	Calcium 0.9%
ppm		g		g		g
			Adolescent			
0	23	48.6 ± 1.1[2ef]	21	51.4 ± 1.1[abcde]	20	49.4 ± 1.1[de]
1	20	46.6 ± 1.2[fgh]	21	49.1 ± 1.1[e]	20	50.6 ± 1.2[bcde]
5	18	39.6 ± 1.2[j]	17	43.3 ± 1.3[hi]	17	45.4 ± 1.3[fghi]
10	15	34.0 ± 1.4[k]	22	33.4 ± 1.1[k]	20	34.6 ± 1.2[k]
			Mature			
0	16	51.3 ± 1.2[abcde]	18	53.1 ± 1.2[abc]	16	54.4 ± 1.3[a]
1	20	51.7 ± 1.2[abcde]	15	53.6 ± 1.3[ab]	16	52.7 ± 1.3[abcd]
5	16	48.6 ± 1.2[ef]	19	50.0 ± 1.2[cde]	13	48.4 ± 1.4[efg]
10	20	39.4 ± 1.1[j]	17	43.2 ± 1.2[i]	16	45.2 ± 1.2[ghi]

[1] Least-Squares Means ± SE.

[2] Means with different superscripts differ at the $p < 0.05$ level of significance.

Results of this study indicate 5 or 10 ppm Cd affects the reproductive performance of adolescent dams at all levels of dietary Ca. However, the reproductive performance of mature dams is affected only by 10 ppm Cd intake at the 0.3 and 0.6% dietary Ca levels but not at the 0.9% Ca level. The high-Ca diet did have a protective effect against 10 ppm Cd in the mature dams. Two factors may account for the lack of the protective effect of the high-Ca diet on the weaning weights of the offspring of adolescent dams. The mature dams had been consuming the high-Ca diets for 128 days compared to only 73 days for the adolescent dams. Also, the adolescent dams were maturing and needed the additional Ca in the diet for their own growth. Although Cd treatment affected the offspring of both the adolescent and mature dams, the effect of additional Cd was greater at a lower intake of Cd in the adolescent dams than it was in the mature dams.

The increase in Ca from 0.6 to 0.9% of the diet of the mature dams appeared the counteract the deliterious effect of 10 ppm Cd in the drinking water. However, increasing the dietary level of Ca in the diet of the adolescent dams did not appear to affect the average weaning weights of their offspring.

B. PLASMA CALCIUM AND PHOSPHORUS

Although plasma Ca and P concentrations usually are maintained within a very narrow range, changes in their concentrations can indicate disturbed parathyroid or kidney function. Parathyroid hormone raises the serum Ca and lowers the serum P and causes release of Ca

and P from the bone when dietary intake of Ca is inadequate. In hyperparathyroidism, serum levels of Ca rise with a concomitant fall in serum P. Also, in renal failure, plasma P is elevated (60).

Cadmium treatment affected the plasma P concentration but not the plasma Ca concentration or the Ca-P ratio (Table A-3, Appendix). Calcium treatment affected the plasma Ca and P concentrations; therefore the plasma Ca-P ratio was affected also. Age significantly affected only the plasma Ca concentration whereas parity affected only the plasma P concentration.

The interaction of Ca and parity affected all three parameters but the interaction of age and parity affected only the plasma Ca and P concentrations. The three-way interaction of Cd, Ca and age indicated a significant difference in the Ca-P ratios and the interaction of Cd, age and parity significantly affected the plasma Ca concentration and the Ca-P ratio.

Effect of Cadmium on Plasma Calcium and Phosphorus Concentrations and the Calcium-Phosphorus Ratio

Investigators have not reached a concensus on the effect of Cd on rats fed Ca-deficient diets. Results of a study by Itokawa and colleagues (21) using male rats treated with 50 ppm Cd indicated serum Ca concentrations were decreased in rats consuming a Ca-deficient diet as compared to those consuming a diet adequate in Ca. However, in a subsequent study, Itokawa and co-workers (22) found no difference in the serum Ca concentration when female rats treated with 100 ppm Cd were fed diets either deficient or adequate in Ca.

The present study also showed that Cd treatment did not affect the plasma Ca or Ca-P ratio but that 5 and 10 ppm Cd in the drinking water significantly increased the plasma P concentration from 5.09 mg/dl at 0 ppm Cd to 5.60 mg/dl at 10 ppm Cd (Table 7). Itokawa and co-workers (21, 22) also found an increased serum P concentration in Cd-treated (50-100 ppm) rats. They attributed the increase in serum P concentration to impaired renal function of the damaged kidney. Results of the present study also showed that an increase in the plasma P concentration occurred when as little as 5 ppm Cd was added to the drinking water.

A visual inspection of the kidneys of the dams in the present study suggested renal impairment in the Cd treated rats. The tubule area of many kidneys in the rats treated with 10 ppm Cd were eroded thus suggesting extensive kidney damage. Although renal function tests were not performed, the visual inspection of the kidney and the demonstrated rise in the plasma P concentration suggests renal impairment in the dams treated with 5 or 10 ppm Cd.

Effect of Calcium on Plasma Calcium and Phosphorus Concentrations and the Calcium-Phosphorus Ratio

The Ca level of the diet significantly affected the plasma Ca concentration as shown in Table 7. The low-Ca diet significantly reduced the plasma Ca concentration to 6.58 mg/dl while the high-Ca diet increased the plasma Ca concentration to 7.35 mg/dl as compared to the plasma Ca concentration of 6.82 mg/dl in rats fed the normal-Ca level. The low-Ca diet did not affect the plasma P concentration but

Table 7

Effect of cadmium, calcium, age of dam and parity on the plasma calcium and phosphorus concentrations and the calcium-phosphorus ratio[1]

Treatment	Number of dams	Calcium	Phosphorus	Ca-P ratio
		mg/dl	mg/dl	
ppm			Cadmium	
0	114	6.85 ± 0.08[2a]	5.09 ± 0.11[a]	1.45 ± 0.05[a]
1	112	6.89 ± 0.08[a]	5.13 ± 0.11[a]	1.48 ± 0.05[a]
5	100	6.92 ± 0.09[a]	5.47 ± 0.11[b]	1.33 ± 0.05[a]
10	110	6.99 ± 0.08[a]	5.60 ± 0.11[b]	1.38 ± 0.05[a]
%			Calcium	
0.3	144	6.58 ± 0.07[c]	5.81 ± 0.09[c]	1.18 ± 0.04[c]
0.6	154	6.82 ± 0.07[d]	5.57 ± 0.09[c]	1.26 ± 0.04[c]
0.9	138	7.35 ± 0.07[e]	4.59 ± 0.10[d]	1.79 ± 0.04[d]
			Age of dam	
Adolescent	235	6.74 ± 0.06[f]	5.29 ± 0.07[e]	1.39 ± 0.03[e]
Mature	201	7.09 ± 0.06[g]	5.35 ± 0.08[e]	1.43 ± 0.04[e]
			Parity	
One	224	7.00 ± 0.06[h]	5.50 ± 0.07[f]	1.41 ± 0.03[f]
Four	212	6.83 ± 0.06[h]	5.14 ± 0.08[g]	1.41 ± 0.03[f]

[1]Least-Squares Means ± SE.

[2]Means within the same column within each subgroup with different superscripts differ at the $p < 0.05$ level of significance.

there was a significant depression in plasma P concentration when dietary Ca intake was increased to 0.9% of the diet. The increase in plasma Ca concentration with the concomitant decrease in plasma P concentration significantly altered the Ca-P ratio for dams fed the 0.9% Ca diet. Some of the dams may have had renal dysfunction as noted previously. The rise in the plasma Ca concentration and fall of the plasma P concentration may have been related to this condition. However, since neither kidney function nor plasma parathyroid hormone were measured, no definite reasons for the different plasma concentration can be stated.

Effect of Age of Dam and Parity on Plasma Calcium and Phosphorus Concentrations and the Calcium-Phosphorus Ratio

The age of the dam had no effect on the plasma P or the Ca-P ratio (Table 7). However, the mature dams had significantly more Ca in the plasma than did the adolescent dams. This may be due to the increased need for Ca by the adolescent dams for bone growth.

Parity of the dams did not affect the plasma Ca or Ca-P ratio (Table 7). Plasma P was significantly reduced after four pregnancies.

Interaction of Calcium and Parity on the Plasma Calcium and Phosphorus Concentrations and the Calcium-Phosphorus Ratio

There was a significant interaction effect between the dietary Ca level and the number of times a dam had been pregnant on the plasma Ca and P concentrations and the Ca-P ratio (Table 8). For the first pregnancy, the plasma Ca concentration of dams fed the low-Ca diet

Table 8

Interaction of calcium and parity on the plasma calcium and phosphorus concentrations and the calcium-phosphorus ratio[1]

Pregnancy	Treatment Ca	Number of dams	Calcium	Phosphorus	Ca-P ratio
	%		mg/dl	mg/dl	
One					
	0.3	72	6.63 ± 0.10^{2cd}	6.12 ± 0.13^{a}	1.13 ± 0.06^{d}
	0.6	79	6.79 ± 0.10^{cd}	5.84 ± 0.13^{ab}	1.20 ± 0.06^{cd}
	0.9	73	7.57 ± 0.10^{a}	4.56 ± 0.13^{d}	1.90 ± 0.06^{a}
Four					
	0.3	72	6.53 ± 0.10^{d}	5.50 ± 0.13^{bc}	1.24 ± 0.06^{cd}
	0.6	75	6.84 ± 0.10^{bc}	5.30 ± 0.13^{c}	1.31 ± 0.06^{c}
	0.9	65	7.13 ± 0.11^{b}	4.61 ± 0.14^{d}	1.68 ± 0.06^{b}

[1]Least-Square Means ± SE.

[2]Means within the same column with different superscripts differ at the $p < 0.05$ level of significance.

was not significantly different from that of dams fed the normal-Ca diet but by the fourth pregnancy, the plasma Ca concentration of the dams fed the low-Ca diet was significantly lower than that of dams fed the normal-Ca diet. After the first pregnancy, an increase in the dietary Ca from 0.6 to 0.9% increased the plasma Ca concentration, decreased the plasma P concentration and increased the Ca-P ratio. By the completion of the fourth lactation period, the plasma Ca concentrations were not significantly different in animals fed the normal or high-Ca diet but the plasma P concentration was still decreased in animals fed high-Ca diets. As the plasma Ca concentration increased and the plasma P concentration decreased there was a significant shift in the Ca-P ratio. The increase in the Ca-P ratio in dams fed the high-Ca diet reflects these changes.

All diets contained the same level of P but varied in Ca content which resulted in diets with different Ca-P ratios. The Ca-P ratio of the low-Ca diet was not as different from the ratio in the normal-Ca diet as was the ratio in the high-Ca diet. The depression of the plasma P concentration with the increase in plasma Ca concentration may be the result of insufficient dietary P to maintain the normal Ca-P ratio.

Interaction of Age and Parity on the Plasma Calcium and Phosphorus Concentrations

There was a significant interaction between the age and parity of the dams on the plasma Ca and P concentrations (Table 9). At the completion of the first pregnancy there was no difference in the plasma

Table 9

Interaction of age of dam and parity on the plasma calcium and phosphorus concentrations[1]

Pregnancy	Number of dams	Calcium	Phosphorus
		mg/dl	mg/dl
One			
Adolescent	120	7.00 ± 0.08[2a]	5.41 ± 0.11[ab]
Mature	104	7.00 ± 0.08[a]	5.30 ± 0.11[b]
Four			
Adolescent	115	6.48 ± 0.08[b]	5.60 ± 0.10[a]
Mature	97	7.19 ± 0.09[a]	4.98 ± 0.11[c]

[1] Least-Square Means ± SE.

[2] Means within the same column with different superscripts differ at the $p < .05$ level of significance.

Ca concentrations of the adolescent and the mature dams. However, by the completion of the fourth pregnancy, the plasma Ca concentration of the mature dams had not changed but the plasma Ca concentration of the adolescent dams had decreased significantly.

After the first pregnancy the P concentration of the plasma was not affected by the age of the dam and by completion of the fourth pregnancy the plasma P concentration of the adolescent dams was not different from that of adolescent dams after the first pregnancy. In contrast, the plasma P concentration of the mature dams after four pregnancies had decreased significantly from that measured after the first pregnancy and from that measured in the adolescent dams after the fourth pregnancy.

Interaction of Cadmium, Calcium and Age of Dam on the Plasma Calcium-Phosphorus Ratio

There was a significant interaction among Cd, Ca and the age of dam on the plasma Ca-P ratio (Table 10). The Ca-P ratio was significantly higher in both adolescent and mature dams fed 0.9% Ca and 0 or 1 ppm Cd than dams fed 0.3 or 0.6% Ca diets. Increasing Cd levels in both adolescent and mature dams did not affect the Ca-P ratio in animals fed the low-Ca or normal-Ca diets. Adolescent and mature dams treated with 5 ppm Cd and the high-Ca diet had lower Ca-P ratios than did the dams treated with 0 or 1 ppm Cd. Adolescent dams fed 10 ppm Cd and the high-Ca diet had a low plasma Ca-P ratio but in mature dams fed this regimen the Ca-P ratio was increased. The plasma Ca-P ratios of some of the dams fed the high- Ca diet were

Table 10

Interaction of cadmium, calcium and age of dam on the plasma calcium-phosphorus ratio[1]

Cadmium	Number of dams	Calcium 0.3%	Number of dams	Calcium 0.6%	Number of dams	Calcium 0.9%
ppm			Adolescent			
0	23	1.09 ± 0.11[2g]	21	1.28 ± 0.12[fg]	21	1.88 ± 0.11[bc]
1	20	1.14 ± 0.12[g]	21	1.25 ± 0.11[fg]	20	2.23 ± 0.12[a]
5	18	1.24 ± 0.12[fg]	17	1.25 ± 0.14[fg]	17	1.54 ± 0.13[def]
10	15	1.09 ± 0.14[g]	22	1.13 ± 0.11[g]	20	1.55 ± 0.12[def]
			Mature			
0	16	1.37 ± 0.13	18	1.33 ± 0.12	15	1.77 ± 0.14
1	20	1.22 ± 0.11[fg]	15	1.34 ± 0.14[fg]	16	1.72 ± 0.13[cde]
5	16	1.16 ± 0.13[g]	19	1.28 ± 0.12[fg]	13	1.56 ± 0.14[cdef]
10	16	1.18 ± 0.12[g]	21	1.22 ± 0.13[fg]	16	2.09 ± 0.13[ab]

[1] Least-Squares Means ± SE.

[2] Means with different superscripts differ at the $p < 0.05$ level of significance.

very high which affected the average ratio for the group. It should be noted that the level of dietary P was 0.5% in all diets whereas the level of Ca varied thus the Ca-P ratio of the diets were different. This may have resulted in a change in the plasma concentration of Ca and P thus the Ca-P ratio was disturbed.

Interaction of Cadmium, Age of Dam and Parity on the Plasma Calcium Concentration and the Calcium-Phosphorus Ratio

There was a significant interaction among Cd treatment, age of dam and parity on the plasma Ca (Table 11) and the Ca-P ratio (Table 12). After four pregnancies, plasma Ca concentration was lower at all Cd levels in adolescent dams compared to the value after the first pregnancy except for dams treated with 10 ppm Cd. In mature dams plasma Ca concentration was not changed by increasing the number of pregnancies except in dams treated with 5 ppm Cd. Although there was a significant interaction on the Ca-P ratio there were no definite trends.

C. BONE CALCIUM AND PHOSPHORUS

The quantity of minerals in the bone depends on many factors. A low dietary intake of Ca has been shown to reduce bone Ca (23). Cadmium ingestion appears to reduce Ca absorption from the gastro-intestinal tract (4). Also, many studies indicate Cd ingestion seriously affects bone mineral content either by decreasing mineralization or increasing resorption (4, 12, 23, 25). Mineralization of the bone also has been related to the age and parity of the female (1).

Table 11

Interaction of cadmium, age of dam and parity on the plasma calcium concentration[1]

Cadmium	Adolescent		Mature	
	Number of dams	Calcium	Number of dams	Calcium
ppm		mg/dl		mg/dl
		Pregnancy one		
0	30	6.88 ± 0.16[2abc]	25	6.97 ± 0.17[abc]
1	30	6.99 ± 0.16[abc]	27	7.09 ± 0.16[ab]
5	30	7.19 ± 0.16[ab]	26	6.61 ± 0.16[d]
10	30	6.91 ± 0.15[abc]	26	7.32 ± 0.17[a]
		Pregnancy four		
0	35	6.43 ± 0.14[d]	24	7.23 ± 0.18[a]
1	31	6.28 ± 0.15[d]	24	7.20 ± 0.17[ab]
5	22	6.55 ± 0.19[cd]	22	7.32 ± 0.18[a]
10	27	6.73 ± 0.17[bc]	27	7.01 ± 0.16[abc]

[1]Least-Squares means ± SE.

[2]Means with different superscripts differ at the $p < 0.05$ level of significance.

Table 12

Interaction of cadmium, age of dam and parity on the plasma calcium-phosphorus ratio[1]

Cadmium	Adolescent		Mature	
	Number of dams	Ca-P ratio	Number of dams	Ca-P ratio
ppm		Pregnancy one		
0	30	1.53 ± 0.10^{abcd}	25	1.39 ± 0.10^{bcde}
1	30	1.56 ± 0.10^{abc}	27	1.42 ± 0.10^{bcde}
5	30	1.28 ± 0.10^{de}	26	1.20 ± 0.10^{e}
10	30	1.22 ± 0.09^{e}	26	1.72 ± 0.10^{a}
		Pregnancy four		
0	35	1.31 ± 0.09^{cde}	24	1.59 ± 0.11^{ab}
1	31	1.52 ± 0.09^{abcd}	24	1.43 ± 0.10^{abcde}
5	22	1.41 ± 0.12^{bcde}	22	1.45 ± 0.11^{abcde}
10	27	1.29 ± 0.10^{cde}	27	1.28 ± 0.10^{de}

[1]Least-Squares Means ± SE.

[2]Means with different superscripts differ at the $p < 0.05$ level of significance.

Cadmium and Ca treatment significantly affected the bone Ca and P content of the femur (Table A-4, Appendix). Age of the dam had no effect on Ca and P content but parity significantly affected the bone P content and the Ca-P ratio. There were three significant interactions. The interaction of Cd and Ca affected the bone Ca and P content as well as the Ca-P ratio. A Cd and parity interaction was present only for the bone P concentration and there was a significant interaction between age and parity for both bone Ca and P content.

Effect of Cadmium on Bone Mineral Content

Cadmium treatment adversely affected both the Ca and P content of the femur (Table 13). Cadmium levels of 1, 5 and 10 ppm significantly reduced femur Ca. Femurs of dams given no Cd contained 22.66 mg Ca/100 mg of dry bone but when 10 ppm Cd was added to the drinking water, femur Ca was reduced to 21.55 mg/100 mg dry bone.

The present work agrees with that of Washko and Cousins (23) who found decreased bone Ca in rats fed a low-Ca diet. Bone ash was decreased further when 25 ppm Cd was added to the drinking water of young growing rats fed the low-Ca diets. They attributed the decreased bone ash in rats fed low-Ca diets to mobilization of bone Ca to compensate for a low-Ca dietary intake. They also suggested Cd had an effect on Ca metabolism which decreases bone ash beyond that produced by a low-Ca diet. Their work confirms that of other investigators but all agree the mechanism of Cd involvement in bone metabolism has not been elucidated.

Table 13

Effect of cadmium, calcium, age of dam and parity on the calcium and phosphorus content and the calcium-phosphorus ratio of the femur[1]

Treatment	Number of dams	Calcium	Phosphorus	Ca-P ratio
		mg/100 mg	mg/ 100mg	
ppm			Cadmium	
0	114	22.66 ± 0.12[2a]	10.20 ± 0.05[a]	2.22 ± 0.01[a]
1	112	22.10 ± 0.12[b]	10.15 ± 0.05[a]	2.18 ± 0.01[a]
5	100	21.77 ± 0.13[bc]	9.90 ± 0.06[b]	2.19 ± 0.01[a]
10	110	21.55 ± 0.12[c]	9.79 ± 0.05[b]	2.21 ± 0.01[a]
%			Calcium	
0.3	144	20.88 ± 0.11[d]	9.50 ± 0.04[d]	2.20 ± 0.01[b]
0.6	154	22.51 ± 0.10[e]	10.27 ± 0.04[e]	2.20 ± 0.01[b]
0.9	138	22.68 ± 0.11[e]	10.28 ± 0.05[e]	2.21 ± 0.01[b]
			Age of dam	
Adolescent	235	21.92 ± 0.09[f]	9.98 ± 0.04[f]	2.20 ± 0.01[c]
Mature	201	22.19 ± 0.08[f]	10.05 ± 0.04[f]	2.21 ± 0.01[c]
			Parity	
One	224	22.10 ± 0.08[g]	10.13 ± 0.04[g]	2.19 ± 0.01[d]
Four	212	21.94 ± 0.09[g]	9.90 ± 0.04[h]	2.22 ± 0.01[e]

[1]Least-Squares Means ± SE.

[2]Means within the same column within each subgroup with different superscripts differ at the $p < 0.05$ level of significance.

One ppm Cd had no effect on the P content of the femur (Table 13). An increase of Cd to 5 or 10 ppm significantly decreased the femur P content. Values for femur P ranged from a high of 10.2 mg/100 mg dry bone in animals receiving no Cd to 9.79 mg/100 mg dry bone at 10 ppm Cd. The decrease in femur P with increasing Cd parallels the decrease in the femur Ca thus Cd had no effect on the Ca-P ratio.

Effect of Calcium on Bone Mineral Content

A reduction in dietary Ca from 0.6 to 0.3% of the diet significantly reduced the Ca and P content of the bone but an increase of dietary Ca to 0.9% of the diet did not significantly increase bone Ca or P (Table 13). The reduction in bone ash in femurs of dams fed low-Ca diets agrees with the work of Washko and Cousins (23) who found reduced bone ash in male rats fed low-Ca diets.

Effect of Age of Dam and Parity on Bone Mineral Content

The number of pregnancies did not alter the bone Ca content but bone P was reduced significantly after four pregnancies (Table 13). The decreased bone P with no change in bone Ca altered the Ca-P ratio of the femur resulting in a significantly higher Ca-P ratio between the first and fourth pregnancies. Although the Ca-P ratio was significantly different between the pregnancies, it may be of little biological significance.

The age of the dam did not significantly affect the bone Ca, P or the Ca-P ratio. The age and parity of the dams did not affect the Ca

content of the femur as might be expected. The low dietary Ca level used was deficient only marginally for the pregnant animal since this level was one-half of the recommended amount for pregnant rats. If the dietary level of Ca had been curtailed severely an effect of age of dam and parity probably would have been observed.

In addition, it should be noted that during pregnancy, the rats consumed more food than they did when not pregnant. Since food was allowed ad libitum, the increased food intake resulted in the consumption of additional Ca. The increased absorption of Ca in the pregnant rats fed the low-Ca diet probably compensated for the low-Ca level in the diet. Therefore, a true Ca deficiency probably was not produced.

Interaction of Cadmium and Calcium on the Calcium and Phosphorus Content and the Calcium-Phosphorus Ratio of the Femur

The interaction of dietary Cd and Ca on bone mineral content is shown in Table 14. There was significant interaction between Cd treatment and dietary Ca level.

Consumption of the low-Ca diet resulted in less bone Ca than that found in animals consuming the normal or the high-Ca diets regardless of Cd treatment. Cadmium levels of 1, 5 and 10 ppm significantly reduced the Ca content of the femur in dams fed the normal-Ca diet. When dietary Ca was increased to 0.9% of the diet the effect of Cd was observed only at the 5 and 10 ppm Cd levels. This indicated a protective effect of additional dietary Ca against very low levels of Cd. When the Cd was increased to 10 ppm in the 0.9% Ca treatment group,

Table 14

Interaction of cadmium and calcium on the calcium and phosphorus content and the calcium-phosphorus ratio of the femur[1]

Treatment Cd	Number of dams	Calcium	Phosphorus	Ca-P ratio
ppm		mg/100 mg	mg/100 mg	
			0.3% Calcium	
0	39	21.22 ± 0.21[2ef]	9.66 ± 0.09[fg]	2.20 ± 0.02[ab]
1	40	20.79 ± 0.20[fg]	9.50 ± 0.08[gh]	2.20 ± 0.02[ab]
5	34	20.55 ± 0.22[g]	9.34 ± 0.09[h]	2.20 ± 0.02[ab]
10	31	20.96 ± 0.22[fg]	9.50 ± 0.10[h]	2.21 ± 0.02[ab]
			0.6% Calcium	
0	39	23.31 ± 0.20[a]	10.48 ± 0.09[ab]	2.23 ± 0.02[a]
1	36	22.45 ± 0.21[c]	10.58 ± 0.09[a]	2.13 ± 0.02[c]
5	36	22.27 ± 0.24[cd]	10.17 ± 0.10[cd]	2.20 ± 0.03[ab]
10	43	22.00 ± 0.20[cd]	9.84 ± 0.09[ef]	2.24 ± 0.02[a]
			0.9% Calcium	
0	36	23.46 ± 0.22[a]	10.45 ± 0.09[ab]	2.25 ± 0.02[a]
1	36	23.06 ± 0.21[ab]	10.36 ± 0.09[abc]	2.23 ± 0.02[ab]
5	30	22.50 ± 0.24[bc]	10.27 ± 0.10[bcd]	2.20 ± 0.03[ab]
10	36	21.70 ± 0.21[de]	10.02 ± 0.09[de]	2.17 ± 0.02[bc]

[1]Least-Square Means ± SE.

[2]Means within the same column with different superscripts differ at the $p < 0.05$ level of significance.

femur Ca was reduced to 21.70 mg/100 mg dry bone. This value was not significantly different from the femur Ca measured in dams fed the normal-Ca diet and treated with 5 or 10 ppm Cd or the dams fed the low-Ca diet with no Cd. Piscator and Larson (25) and Washko and Cousins (23) also found decreased bone Ca in male and female rats treated with Cd levels ranging from 2.5 to 25 ppm.

A normal or high level of Ca in the diet helped offset some of the effects of the high-Cd levels but did not completely overcome the effects of the Cd. However, the Ca content in the bone of rats fed the highest (0.9%) Ca and (10 ppm) Cd levels were at least as high as that of rats fed the low-Ca diet containing no Cd.

Table 14 indicates that as the dietary Ca was decreased from 0.6 to 0.3% of the diet, the bone Ca content also decreased. When dietary Ca was increased from 0.6 to 0.9% of the diet, no changes in bone Ca were noted except at the 1 ppm Cd level when bone Ca increased from 22.45 to 23.06 mg/100 mg dry bone.

The P content of femurs of dams fed the no-Cd low-Ca diet was lower than that of animals fed either the normal or high-Ca diet with no Cd (Table 14). Addition of Cd to the low-Ca diet did not affect greatly the bone P content. When dams were fed a normal-Ca diet and 5 or 10 ppm Cd, the femur P content was significantly less than that observed at 0 or 1 ppm Cd. Calcium in the diet above normal levels did not appear to affect the bone P. The only significant decrease in bone P in dams fed the high-Ca diet occurred in animals fed 10 ppm Cd again indicating the high-Ca diet seemed to protect the bone P of

animals treated with 5 ppm Cd. The Ca-P ratio was not significantly reduced except in animals fed the 1 ppm Cd normal-Ca diet and the 10 ppm Cd high-Ca diet. It should be noted the dietary level of P was constant even though the Ca level in the diet varied. This resulted in a different Ca-P ratio in each diet. The fluctuations in the bone P content may be an attempt by the bone to maintain the Ca-P ratio necessary for proper bone formation.

Interaction of Cadmium and Parity on the Phosphorus Content of the Femur

There was an interaction between Cd and parity on the P content of the femur of the dams (Table 15). After the fourth pregnancy, dams treated with 5 or 10 ppm Cd had a lower bone P content than did dams treated with 0 or 1 ppm Cd. No differences were noted between the bone P content of dams treated with any Cd level after the first pregnancy and the dams treated with 0 or 1 ppm Cd at the completion of the fourth pregnancy.

The decrease in the bone P content in animals consuming the high- Cd levels after the fourth pregnancy may be due to insufficient amounts of P available for bone formation. Dams treated with high-Cd levels for an extended period of time may have increased plasma P levels due to increased bone resorption. It is also possible that Cd caused demineralization of bone resulting in the release of bone P into plasma. The increased plasma P concentration observed with increasing levels of Cd (Table 7, p. 60) would support this idea.

Table 15

Interaction of cadmium and parity on the phosphorus content of the femur[1]

Parity	Cadmium	Number of dams	Phosphorus
	ppm		mg/100 mg bone
One			
	0	55	10.15 ± 0.08[2ab]
	1	57	10.22 ± 0.07[ab]
	5	56	10.12 ± 0.07[ab]
	10	56	10.02 ± 0.07[b]
Four			
	0	59	10.25 ± 0.07[a]
	1	55	10.08 ± 0.07[ab]
	5	44	9.73 ± 0.08[c]
	10	54	9.56 ± 0.08[c]

[1]Least-Squares Means ± SE.

[2]Means with different superscripts differ at the $p < 0.05$ level of significance.

Interaction of Age and Parity on the Calcium and Phosphorus Content of the Femur

The interaction of age and parity on bone mineral content suggests mature dams who had been pregnant only once had femurs with the highest Ca content (Table 16). After four pregnancies the femur Ca was decreased in the mature dams to a value comparable to that of the adolescent dams after the first or fourth pregnancy. The adolescent dams that had been pregnant only once had femurs whose Ca content was not significantly different from the adolescent dams which had been pregnant four times. The decrease in bone Ca in the mature dams after four pregnancies agrees with results of Sugawara and Sugawara (33) who found decreased Ca in bone ash of dams pregnant two times. The low Ca content of 21.77 mg/100 mg of dry femur of the adolescent dam as compared to 22.08 mg/100 mg of dry bone of mature dams after one pregnancy may be due to the immaturity of the bone of younger animals. By the completion of four pregnancies, the adolescent dam had reached biological maturity; therefore, bone ash did not differ significantly from her mature counterpart.

The P content of the femur of adolescent dams did not change due to parity. However, the mature dams after the first pregnancy had a higher femur P than did the adolescent dams but a lower P content after four pregnancies.

D. BONE DENSITY AND BONE STRENGTH

Bone quality may be measured by chemical analysis or by mechanical tests. The quantity of bone ash, particularly Ca and P may be

Table 16

Interaction of age and parity on the calcium and phosphorus content of the femur[1]

Pregnancy	Number of dams	Calcium	Phosphorus
		mg/100 mg bone	mg/100 mg bone
One			
Adolescent	120	21.77 ± 0.12[2a]	9.96 ± 0.05[a]
Mature	104	22.43 ± 0.12[b]	10.29 ± 0.05[b]
Four			
Adolescent	115	22.08 ± 0.12[a]	10.00 ± 0.05[a]
Mature	97	21.81 ± 0.13[a]	9.80 ± 0.06[c]

[1]Least-Square Means ± SE.

[2]Means within each column with different superscripts differ at the $p < 0.05$ level of significance.

determined to assess the degree of mineralization of the bone. Density and strength measurements also may be made in an attempt to quantify bone quality.

Both Cd and Ca treatment of the dams significantly affected the density and strength of the femur (Table A-5, Appendix). Density but not strength was affected significantly by the age of the dam but parity had no effect on either parameter.

There was a significant interaction between Cd and Ca on bone density but not on bone strength. Interactions between Cd and parity, and age and parity affected both bone density and bone strength. The three-way interaction of Cd, age and parity was significant only for bone strength.

Effect of Cadmium on the Bone-Density Index

Data on the bone-density index measurements are found in Table 17. The bone-density index decreased from 0.94 in dams given no Cd to 0.80 in dams treated with 10 ppm Cd. There was no difference in the bone-density index of femurs of rats consuming 0 or 1 ppm Cd in the drinking water and no difference in the density of femurs of rats receiving 5 or 10 ppm Cd. However, there was a significant difference between the animals receiving 0 or 1 and 5 or 10 ppm Cd. The reduced bone-density index in animals receiving the higher Cd levels is compatible with the low Ca and P content of the bone with increased Cd intake (Table 13, p. 71). The low bone-density index observed in dams treated with 5 or 10 ppm Cd may be due to

Table 17

Effect of cadmium, calcium and age of dam on the bone-density index and bone strength of the femur[1]

Treatment	Number of dams	Bone-density index	Bone strength
			lbs of force
ppm		Cadmium	
0	114	0.94 ± 0.01[2a]	19.15 ± 0.32[a]
1	112	0.92 ± 0.01[a]	19.30 ± 0.32[a]
5	100	0.84 ± 0.01[b]	16.74 ± 0.34[b]
10	110	0.80 ± 0.01[b]	15.98 ± 0.32[b]
%		Calcium	
0.3	144	0.74 ± 0.01[c]	14.30 ± 0.28[c]
0.6	154	0.92 ± 0.01[d]	19.07 ± 0.28[d]
0.9	138	0.95 ± 0.01[d]	20.01 ± 0.29[d]
		Age of dam	
Adolescent	235	0.86 ± 0.01[e]	17.75 ± 0.24[e]
Mature	201	0.88 ± 0.01[f]	17.83 ± 0.22[e]

[1] Least-Squares Means ± SE.

[2] Means within the same column within each subgroup with different superscripts differ at the $p < 0.05$ level of significance.

inadequate absorption of Ca needed for bone mineralization or to the effect of Cd on bone formation or resorption.

Effect of Dietary Calcium on the Bone-Density Index

The dietary Ca level also affected the bone-density index (Table 17). A low-Ca intake resulted in a bone-density index significantly lower than that achieved by animals consuming the two higher Ca levels. Increasing the dietary Ca from 0.6 to 0.9% did not increase significantly bone quality as measured by the bone-density index. These findings also parallel the results of the bone Ca and P analysis which indicated reduced bone Ca and P in dams consuming low- Ca diets (Tables 13, p. 71, and 17).

Effect of Age of Dam on the Bone-Density Index

The age of the animal at first breeding affected the bone-density index of the femur (Table 17). Mature animals had a significantly higher degree of bone mineral content as measured by the bone-density index than did those animals who were bred as adolescents which confirms the findings of the bone Ca analysis. The lower bone density in adolescent dams may be due to the immaturity of their bone.

Effect of Cadmium on Bone Strength

Bone strength is an indication of bone quality and may be measured as the pounds of force required to break the bone (breaking load). Results (Table 17) of bone strength measurements of animals fed the varying Cd treatments parallel those of the bone-density index

and bone mineral content. One ppm Cd in the drinking water did not affect adversely the strength of the bone. However, when Cd was increased to 5 or 10 ppm a significant loss of bone strength was observed. The decrease in bone strength is due probably to the decreased bone mineral content of the femur.

Effect of Dietary Calcium on Bone Strength

Dietary Ca also affected the strength of the bone. In rats fed the low-Ca diet only 14.30 pounds of force were required to break the bone (Table 17). With an increase in dietary Ca to the normal level, an additional 4.77 pounds of force were required to break the bone. There was no significant difference in the force required to break the femur when Ca was increased from 0.6 to 0.9% of the diet. These results are in agreement with the decreased bone mineral and density found in dams fed the low-Ca diet.

It appears from the data presented that dams fed diets low in Ca have lower bone mineral, density and strength than do dams fed diets adequate in Ca. Increasing the dietary Ca above the recommended level did not increase the bone mineral, density or strength. Also, age and parity of the dam did not affect bone strength.

Interaction of Cadmium and Dietary Calcium on the Bone-Density Index

The bone-density index of the femur of dams fed the low-Ca diet was lower than the bone-density index of the dams fed the adequate or high-Ca diet at any Cd level (Table 18). Femurs of dams fed the high-Ca no Cd diet were significantly more dense than femurs of dams

Table 18

Interaction of cadmium and calcium on the bone-density index of the femur[1]

Cadmium	Number of dams	Calcium 0.3%	Number of dams	Calcium 0.6%	Number of dams	Calcium 0.9%
ppm						
0	39	0.76 ± 0.02[2e]	39	0.99 ± 0.02[b]	36	1.05 ± 0.02[a]
1	40	0.77 ± 0.02[e]	36	0.98 ± 0.02[bc]	36	0.99 ± 0.02[b]
5	34	0.71 ± 0.02[f]	36	0.86 ± 0.02[d]	30	0.93 ± 0.02[c]
10	31	0.70 ± 0.02[f]	43	0.84 ± 0.02[d]	36	0.85 ± 0.02[d]

[1] Least-Square Means ± SE.

[2] Means with different superscripts differ at the $p < 0.05$ level of significance.

from in any other treatment group. The density of the femurs of dams treated with the high-Ca diet and 5 ppm Cd was greater than the density of femurs of dams fed the normal-Ca diet and 5 ppm Cd. However, there was no significant difference between the density of the femurs of dams treated with 10 ppm Cd and the normal or high-Ca diet whereas dams fed the 0.3% Ca diet and consuming 5 or 10 ppm Cd had the lowest bone density of any group. The high-Ca diet minimized the depressing effects of the 5 ppm Cd treatment but the effects of the 10 ppm Cd treatment were not reduced.

Interaction of Cadmium and Parity on the Bone-Density Index and Bone Strength

An interaction exists between Cd treatment and parity on both the bone-density index and bone strength of dams (Table 19). After the first pregnancy, increasing the Cd from 0 to 1 ppm Cd did not affect bone density but at 5 or 10 ppm Cd bone density was decreased. After the fourth pregnancy as little as 1 ppm Cd depressed bone density. The decrease in bone density with increasing Cd treatment was observed to a greater extent after the fourth pregnancy than after the first. The decrease in bone density after the first pregnancy was only 0.07 units when Cd treatment was increased from 1 ppm to 10 ppm. By the fourth pregnancy, the difference in the bone density increased to 0.16 units. In animals fed 0 ppm Cd, there was an increase in bone density with an increase in the number of pregnancies. In animals fed 1 or 5 ppm Cd there was no change in the bone density as the number of pregnancies increased but in

Table 19

Interaction of cadmium and parity on the bone-density index and bone strength of the femur[1]

Cadmium	Number of dams	Bone-density index	Bone strength
ppm			lbs of force
		Pregnancy one	
0	55	0.98 ± 0.01^{c}	18.12 ± 0.45^{a}
1	57	0.93 ± 0.01^{a}	18.51 ± 0.44^{a}
5	56	0.82 ± 0.02^{b}	17.09 ± 0.44^{b}
10	56	0.77 ± 0.02^{d}	17.60 ± 0.44^{a}
		Pregnancy four	
0	59	0.89 ± 0.01^{2a}	20.18 ± 0.44^{c}
1	55	0.90 ± 0.01^{a}	20.09 ± 0.45^{c}
5	44	0.85 ± 0.01^{b}	16.39 ± 0.52^{b}
10	54	0.83 ± 0.01^{b}	14.36 ± 0.47^{d}

[1] Least-Square Means ± SE.

[2] Means within each column with different superscripts differ at the $p < 0.05$ level of significance.

animals consuming 10 ppm Cd the bone-density index after four pregnancies was decreased markedly from the bone density measured after the first pregnancy.

After four pregnancies, bone strength increased in animals receiving 0 and 1 ppm Cd but there was no change in bone strength from first to fourth pregnancy in animals receiving 5 ppm Cd. Ten ppm Cd significantly reduced bone strength after four consecutive pregnancies. Results of bone breaking measurements support those observed in bone density measurements (Table 19). Bone strength decreased from 18.12 pounds of force at 0 ppm Cd to 17.09 pounds of force at 5 ppm Cd after the first pregnancy. By the fourth pregnancy, the bone strength decreased from 20.18 pounds of force at 0 ppm Cd to 14.36 pounds of force at 10 ppm Cd, a decrease of 5.82 pounds.

Dams consuming no or very low levels of Cd appeared to utilize their dietary Ca to increase bone density and strength with an increase in age regardless of the number of pregnancies. Administration of 5 ppm Cd resulted in no differences in bone density and strength after one or four pregnancies whereas the administration of 10 ppm Cd decreased bone density and strength after four pregnancies as compared to the values after one pregnancy. The 10 ppm Cd level was high enough to either interfere with Ca absorption, bone formation or resorption or perhaps cause Cd and P loss through damaged kidneys.

The depressing effect of Cd on the bone-density index and bone strength particularly after four pregnancies in this animal study resemble the observations on the bones of multiparous Japanese women suffering itai-itai disease (4). These women had numerous bone fractures after chronic ingestion of Cd which appeared to be the result of weak and brittle bones caused by decalification of the skeleton.

Interaction of Age of Dam and Parity on Bone-Density Index and Bone Strength

The bone-density index of the mature rats did not differ significantly from the first to the fourth pregnancy (Table 20). However, the bone-density index of the adolescent group was only 0.84 at the completion of the first pregnancy but was increased to 0.87 by the end of the fourth pregnancy. This may indicate the adolescent rat was able to increase the mineralization per unit area of the bone even though she had multiple pregnancies.

The effect of age of the dam and parity on bone strength is not defined as clearly as that for the bone-density index. After the first pregnancy, the femurs of mature dams required more force to break them than did the femurs of the adolescent dams. In contrast, after the fourth pregnancy the adolescents had stronger bones and the bone strength of mature dams had decreased from 18.43 pounds of force after the first pregnancy to 17.22 pounds of force after the fourth pregnancy. The reason for this is unclear since it is not consistent with the results observed for bone density measurements or bone mineral analysis.

Table 20

Interaction of age of dam and parity on the bone-density index and bone strength of the femur[1]

Age	Number of dams	Bone-density index	Bone strength
			lbs of force
		Pregnancy one	
Adolescent	120	0.84 ± 0.01[2a]	17.23 ± 0.30[a]
Mature	104	0.89 ± 0.01[b]	18.43 ± 0.32[b]
		Pregnancy four	
Adolescent	115	0.87 ± 0.01[b]	18.29 ± 0.32[b]
Mature	97	0.88 ± 0.01[b]	17.22 ± 0.34[a]

[1] Least-Squares Means ± SE.

[2] Means with different superscripts within each column differ at the $p < 0.05$ level of significance.

Interaction of Cadmium, Age of Dam and Parity on Bone Strength

No significant differences were noted in the bone strength of the femur of adolescent dams after one pregnancy when treated with any Cd level (Table 21). The bone strength of the mature dams treated with 5 ppm Cd was significantly less than that of mature dams treated with 0 or 1 ppm Cd. There was no difference in the strength of the femur of mature dams treated with 0, 1 or 10 ppm Cd after one pregnancy.

By the completion of the fourth pregnancy, the femurs of the adolescent and mature dams treated with 0 or 1 ppm Cd were significantly stronger than those of the dams treated with 5 or 10 ppm Cd. The femurs of the mature dams treated with 5 ppm Cd were stronger than those of the dams treated with 10 ppm Cd after four pregnancies.

The femurs of the mature dams treated with 0 or 1 ppm Cd after the first pregnancy were stronger than any of the femurs of the adolescent dams regardless of Cd treatment. Femurs of the mature dams treated with 5 or 10 ppm Cd were not significantly different from the femurs of the adolescent dams at all Cd treatment levels. After four pregnancies, the femurs of adolescent dams treated with 0 or 1 ppm Cd were equal in strength to the femurs of the mature dams given no Cd. The strength of the femurs of dams treated with 5 ppm Cd were the same for both age groups at the completion of the fourth pregnancy. There was no difference in the strength of the femurs for the adolescent and mature dams treated with 10 ppm Cd after four pregnancies.

Table 21

Interaction of cadmium, age of dam and parity on the bone strength of the femur[1]

Cadmium	Pregnancy one		Pregnancy four	
	Number of dams	Bone strength	Number of dams	Bone strength
ppm		lbs of force		lbs of force
		Adolescent		
0	30	16.65 ± 0.61[2ef]	35	20.91 ± 0.56[ab]
1	30	17.59 ± 0.60[de]	31	21.17 ± 0.59[a]
5	30	17.32 ± 0.60[de]	22	16.06 ± 0.74[ef]
10	30	17.32 ± 0.60[de]	27	15.02 ± 0.68[fg]
		Mature		
0	25	19.58 ± 0.66[abc]	24	19.44 ± 0.69[abc]
1	27	19.43 ± 0.64[bc]	24	19.00 ± 0.68[cd]
5	26	16.85 ± 0.65[e]	22	16.72 ± 0.73[ef]
10	26	17.86 ± 0.65[cde]	27	13.71 ± 0.65[g]

[1] Least-Square Means ± SE.

[2] Means with different superscripts differ at the $p < 0.05$ level of significance.

Results of the bone strength measurements indicate the adolescent dams treated with 0 or 1 ppm Cd were able to increase bone strength even though pregnant four times. The adolescent dams treated with 5 ppm Cd were able to maintain the strength of the femur but the adolescent dams treated with 10 ppm Cd lost bone strength over the course of four pregnancies. The mature dams treated with 0, 1 or 5 ppm Cd were able to maintain their bone strength through four pregnancies. The mature dams, like the adolescent dams, lost bone strength by the completion of the fourth pregnancy when treated with 10 ppm Cd. This indicates the addition of 10 ppm Cd to the drinking water reduces bone strength of multiparous rats, which may be due to inadequate Ca absorption to meet the demands of pregnancy and lactation or to increased resorption of bone Ca to meet these needs.

CHAPTER V

SUMMARY

Relationships among dietary Cd and Ca, age and parity were investigated in the female rat. Reproductive performance, plasma and bone Ca and P concentrations, and bone density and strength were measured in dams of two different ages after one or four pregnancies.

Weanling female rats were fed diets containing one of three Ca levels (0.3, 0.6 or 0.9% of the diet) and one of four Cd levels (0, 1, 5 or 10 ppm) in the drinking water. One-half of each group was bred as adolescent (55 days of age) and the other half as mature (110 days of age) females. One-half of each age group was sacrificed after the first pregnancy/lactation period and the remaining dams sacrificed at the completion of four pregnancy/lactation periods. In all, there were 48 treatment groups. Animals that did not conceive four consecutive times, had stillborn litters or had three or fewer pups three days postpartum were removed from the study.

Reproductive performance was assessed by comparing, by treatment group, the birth and weaning weights of the offspring of adolescent and mature dams from the first and fourth pregnancies. First litter female offspring of dams treated with 5 ppm Cd were significantly smaller at birth than offspring of dams treated with 0, 1 or 10 ppm Cd. First litter male offspring of dams treated with 5 or 10 ppm Cd were significantly smaller than those from dams treated with 0 or 1 ppm Cd. By the fourth pregnancy, only 10 ppm Cd depressed birth weight.

Weaning weights of offspring of dams fed 5 ppm Cd were less than those of offspring of dams fed 0 or 1 ppm Cd regardless of the number of pregnancies. An increase of Cd to 10 ppm in the drinking water of the dams further depressed the weaning weights of the pups of both sexes of both the first and fourth pregnancy.

A diet low in Ca (0.3%) decreased the weaning weight of both male and female offspring but increasing dietary Ca to 0.9% of the diet from the normal level (0.6%) failed to increase the weaning weight of the pups at either the first or fourth pregnancy. The weaning weights of the male offspring of the mature dams treated with 10 ppm Cd were depressed to the same extent as the weaning weights of the adolescent dams treated with 5 ppm Cd regardless of Ca level of the diet. A chronic intake of 10 ppm Cd at any dietary Ca level decreased weaning weights of the male offspring of the adolescent dams after the first pregnancy but not after the fourth pregnancy.

There was a three-way interaction among Cd, Ca and age of dam on weaning weight of the male offspring after the first pregnancy. Pups born to adolescent dams weighed less than did those born to mature dams after the first pregnancy but age did not influence weaning weight of the offspring by the completion of the fourth pregnancy. Pups in each Cd-Ca treatment group from the fourth pregnancy weighed more than pups in the corresponding Cd-Ca treatment group from the first pregnancy regardless of the age of the dam.

Cadmium treatment had no effect on the plasma Ca concentration or the Ca-P ratio but 5 and 10 ppm Cd significantly increased the

plasma P concentration probably due to kidney damage. The Ca level of the diet did affect the plasma Ca and P concentration. The plasma Ca concentration was depressed in dams fed low-Ca diets and increased in dams fed high-Ca diets as compared to the concentration found in rats fed normal-Ca diets. Rats fed high-Ca diets had depressed plasma P concentration. These changes in plasma Ca and P concentrations increased the Ca-P ratio in dams fed high-Ca diets at both the first and fourth pregnancies. Parity did not affect the plasma Ca concentration but the plasma P concentration was significantly lower after four pregnancies. The mature dams had a significantly higher plasma Ca concentration than did the adolescent dams but the plasma P concentration was unaffected.

Bone mineral was adversely affected by 5 or 10 ppm Cd treatment and the low-Ca diet. Dams consuming 10 ppm Cd had femurs with significantly less Ca and P than did those consuming 0 or 1 ppm Cd. Decreasing dietary Ca to 0.3% of the diet significantly decreased the bone Ca and P content but increasing the dietary Ca above the normal level failed to increase bone mineral content. An interaction between Cd and Ca was observed. When the highest-Cd level was fed in conjunction with a low-Ca diet, femur Ca and P content was significantly reduced below the femur mineral content of animals fed normal-Ca diets at any Cd level.

Age did not effect the Ca or P content of the femur when measured as a main effect. However, after one pregnancy, femur Ca was higher in mature dams than it was in adolescent dams but by the

completion of the fourth pregnancy there was no difference in femur Ca. Femur P was significantly lower in mature dams after four pregnancies than it was in the adolescent dams after four pregnancies or in either age group after one pregnancy. Results of bone density and bone strength measurements indicated that 1 ppm Cd had no effect on the bone density or strength but 5 or 10 ppm Cd significantly decreased the bone density and strength. Ten ppm Cd decreased bone density and strength to a greater extent after the fourth pregnancy than after the first pregnancy. This parallels the depressing effect of 5 or 10 ppm Cd on bone mineral content. Dams fed the low Ca (0.3%) diet had decreased bone density and strength as well as decreased Ca and P content but dams fed dietary Ca above normal levels failed to show an increase in these parameters. Adolescent dams had less mineral content, bone density and bone strength than did the mature dams after the first pregnancy. By the completion of the fourth pregnancy, there was no difference in the bone mineral or bone density by age of dam but the femurs of the adolescent dams were stronger than those of the mature dams.

In conclusion, the results of the statistical analysis of the main effects indicated the addition of 5 or 10 ppm Cd to the diet adversely affected reproductive performance, plasma P, bone mineral, density and strength. Dams fed low-Ca diets had poorer reproductive performance, reduced plasma and bone Ca and reduced bone density and strength than did those fed diets containing a normal or high level of Ca. Increasing the dietary Ca from 0.6 to 0.9% of the diet did not

appear to affect reproductive performance, plasma Ca and P, or bone mineral content, density and strength.

The age of the dam affected reproductive performance, plasma Ca and bone density and strength. Adolescent dams had smaller offspring than did the mature dams at the completion of the first pregnancy, an effect that was not present at the completion of the fourth pregnancy. The plasma Ca concentration of the mature dams was higher than that of the adolescent dams but the plasma P concentration was unaffected by age of dam. The bone mineral content and bone strength was not affected by the age of the dam but the bone-density index was significantly less in the adolescent dam. Parity did not affect the plasma Ca concentration but after four pregnancies, the plasma P concentration of the mature dams was significantly lower than that of the adolescent dams. The bone-density index, bone strength and bone Ca content were not affected by parity but after four pregnancies, the femur P content was less than after one pregnancy.

Several interactions were present among Cd, Ca, age and parity. Increasing the dietary Ca from 0.6 to 0.9% of the diet provided a protective effect against Cd toxicity on the outcome of pregnancy and bone quality during pregnancy as measured by bone mineral content, bone-density index and bone strength. Adolescent dams were more affected than their mature counterparts by both a low-Ca intake and by the addition of 5 or 10 ppm Cd to the drinking water. Bone mineral content, density and strength were higher for both adolescent and mature dams when fed a high-Ca diet as compared animals fed to a

low-Ca diet. The high-Ca diet, especially when fed to adolescent dams, appeared to counteract the toxic effects of Cd.

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APPENDIX

Table A-1

Mean squares and significance of F ratios for average birth weights of male and female offspring of dams after the first and fourth pregnancies

Sources of variation	First pregnancy			Fourth pregnancy		
		Males	Females		Males	Females
	df	Mean squares	Mean squares	df	Mean squares	Mean squares
Total	435	0.381	0.390	211	0.848	0.536
Cd	3	1.174*	1.365*	3	6.687***	3.702**
Ca	2	0.139	0.271	2	0.980	0.176
Cd x Ca	6	0.132	0.464	6	0.902	0.524
Age	1	0.775	2.265*	1	0.013	0.004
Cd x Age	3	0.281	0.167	3	0.674	0.669
Ca x Age	2	0.160	0.323	2	0.088	0.031
Cd x Ca x Age	6	0.359	0.422	6	1.150	0.227
Error[1]	412	0.766	0.395	188	0.004	0.509

[1]Error term for Cd, Ca, Cd x Ca, Age, Cd x Age, Ca x Age, Cd x Ca x Age.

* $p < 0.05$.

** $p < 0.001$.

*** $p < 0.0001$.

Table A-2

Mean squares and significance of F ratios for average weaning weights of male and female offspring of dams after the first and fourth pregnancies

Sources of variation	First pregnancy			Fourth pregnancy		
		Males	Females		Males	Females
	df	Mean squares	Mean squares	df	Mean squares	Mean Squares
Total	435	63.769	51.264	211	35.275	32.797
Cd	3	4135.340***	3332.100***	3	834.909***	626.564***
Ca	2	245.907***	113.027*	2	109.027*	89.638*
Cd x Ca	6	10.233	17.724	6	17.503	23.011
Age	1	3387.435***	2547.546***	1	0.004	32.276
Cd x Age	3	198.598**	217.782***	3	48.876	27.242
Ca x Age	2	2.779	0.643	2	2.067	10.945
Cd x Ca x Age	6	62.745*	31.523	6	31.085	29.316
Error[1]	412	26.719	22.061	188	22.688	23.365

[1]Error term for Cd, Ca, Cd x Ca, Age, Cd x Age, Ca x Age, Cd x Ca x Age.

* $p < 0.05$.

** $p < 0.001$.

*** $p < 0.0001$.

Table A-3

Mean squares and significance of F ratios for plasma calcium and phosphorus concentrations and the calcium-phosphorus ratio

Sources of variation	df	Calcium	Phosphorus	Ca-P Ratio
		Mean squares	Mean squares	Mean squares
Total	435	0.870	1.611	0.354
Cd	3	0.792	7.615**	0.558
Ca	2	19.945***	61.568***	15.098***
Cd x Ca	6	0.542	1.608	0.424
Age	1	13.001***	0.243	0.252
Cd x Age	3	1.602	1.442	0.591
Ca x Age	2	0.175	1.275	0.131
Cd x Ca x Age	6	0.776	0.848	0.559*
Parity	1	3.626	15.027**	0.024
Cd x Parity	3	0.688	1.272	0.494
Ca x Parity	2	2.952*	5.307*	1.546*
Cd x Ca x Parity	6	0.504	1.943	0.447
Age x Parity	1	11.421***	7.094*	0.006
Cd x Age x Parity	3	2.164*	1.602	0.913*
Ca x Age x Parity	2	1.118	2.238	1.077
Cd x Ca x Age x Parity	6	0.744	0.886	0.436
Error[1]	388	0.692	1.166	0.354

[1]Error term for Cd, Ca, Cd x Ca, Age, Cd x Age, Ca x Age, Cd x Ca x Age, Parity, Cd x Parity, Ca x Parity, Cd x Ca x Parity, Age x Parity, Cd x Age x Parity, Ca x Age x Parity, Cd x Ca x Age x Parity.

* $p < 0.05$.

** $p < 0.001$.

*** $p < 0.0001$.

Table A-4

Mean squares and significance of F ratios of bone calcium and phosphorus content and the calcium-phosphorus ratio of the femur

Sources of variation	df	Calcium	Phosphorus	Ca-P Ratio
		Mean squares	Mean squares	Mean squares
Total	435	0.026	0.005	0.021
Cd	3	0.262***	0.038***	0.039
Ca	2	1.444***	0.292***	0.004
Cd x Ca	6	0.054*	0.011*	0.055*
Age	1	0.046	0.005	0.009
Cd x Age	3	0.015	0.001	0.013
Ca x Age	2	0.004	0.004	0.010
Cd x Ca x Age	6	0.077	0.006	0.015
Parity	1	0.021	0.042***	0.095*
Cd x Parity	3	0.046	0.018**	0.020
Ca x Parity	2	0.021	0.002	0.012
Cd x Ca x Parity	6	0.003	0.004	0.028
Age x Parity	1	0.223**	0.078***	0.019
Cd x Age x Parity	3	0.021	0.008	0.006
Ca x Age x Parity	2	0.003	0.001	0.012
Cd x Ca x Age x Parity	6	0.014	0.003	0.036
Error[1]	388	0.016	0.003	0.020

[1]Error term for Cd, Ca, Cd x Ca, Age, Cd x Age, Ca x Age, Cd x Ca x Age, Parity, Cd x Parity, Ca x Parity, Cd x Ca x Parity, Age x Parity, Cd x Age x Parity, Ca x Age x Parity, Cd x Ca x Age x Parity.

* $p < 0.05$.

** $p < 0.001$.

*** $p < 0.0001$.

Table A-5

Mean squares and significance of F ratios for bone-density index and bone strength of the femur

Source of variation	df	Bone-density index	Bone strength
		Mean squares	Mean squares
Total	435	0.024	20.402
Cd	3	0.647***	274.465***
Ca	2	1.884***	1329.331***
Cd x Ca	6	0.032*	4.881
Age	1	0.054*	0.768
Cd x Age	3	0.021	6.191
Ca x Age	2	0.011	12.383
Cd x Ca x Age	6	0.006	19.487
Parity	1	0.014	1.503
Cd x Parity	3	0.115***	165.400***
Ca x Parity	2	0.007	7.122
Cd x Ca x Parity	6	0.007	10.211
Age x Parity	1	0.064*	157.142**
Cd x Age x Parity	3	0.005	38.731*
Ca x Age x Parity	2	0.008	16.137
Cd x Ca x Age x Parity	6	0.016	12.187
Error[1]	388	0.012	10.907

[1]Error term for Cd, Ca, Cd x Ca, Age, Cd x Age, Ca x Age, Cd x Ca x Age, Parity, Cd x Parity, Ca x Parity, Cd x Ca x Parity, Age x Parity, Cd x Age x Parity, Ca x Age x Parity, Cd x Ca x Age x Parity.

* $p < 0.05$.

** $p < 0.001$.

*** $p < 0.0001$.

VITA

Beverley Fee Hammond was born in Ypsilanti, Michigan on September 24, 1943. She is the daughter of Irene L. Fee and the late Edwin F. Fee. She attended Barry Community Schools and graduated from Barry High School in Barry, Illinois in May, 1961. The following September, she entered the University of Illinois in Urbana, Illinois and received a Bachelor of Science in Home Economics in June, 1967.

Upon completion of this degree, she entered the United States Army Medical Specialist Corps as a second lieutenant. She completed a dietetic internship at Brook General Hospital, Fort Sam Houston, Texas in August, 1968. During this internship she married Clark Richard Hammond of Decatur, Illinois. In December, 1968, a son Brett Ian was born. In February, 1969 she began dietary consultation for nursing homes and small hospitals in the Austin, Texas area.

In September, 1969 she entered the Graduate School of the University of Texas at Austin majoring in nutrition. While at the University of Texas she was awarded an Allied Health Traineeship. She accepted a position on the Home Economics faculty of Mary Hardin-Baylor College, Belton, Texas in August 1971. She was awarded a Master of Arts degree from the University of Texas at Austin in May, 1972. In May, 1974 a second son, Jeremy Zane was born and a daughter Cary Lyn was born in January, 1976.

In June 1977 she entered The University of Tennessee, Knoxville. During her graduate work she held an appointment as a teaching assistant and served as a Clinical Instructor in the Coordinated Undergraduate Program in Dietetics. In January 1980 she was awarded

a Graduate Laboratory Participantship administered by Oak Ridge Associated Universities to conduct graduate research at the Comparative Animal Research Laboratory, Oak Ridge, Tennessee.

She is a member of the honor societies Omicron Nu, Phi Kappa Phi and Sigma Xi. She is also a member of the American Dietetics Association, American Home Economics Association Institute & Food Technologists and the Society for Nutrition Education.

In September 1982 she accepted the position of Instructor in the Department of Nutrition and Food Science in the College of Home Economics at The Univeristy of Tennessee, Knoxville. In May, 1984 she resigned this position to complete her doctorial degree. Under the direction of Dr. Jane R. Savage she completed the requirements for the Doctor of Philosophy degree in Home Economics with a major in nutrition in June 1985.