

The effect of prenatal protein-energy malnutrition on the development of mandibles and long bones in newborn rats

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1. To evaluate the role of gestational protein-energy malnutrition on fetal hard-tissue growth and metabolism, we measured several variables in the growth centres of mandibles and long bones of newborn rats.
2. Control pups and pups of malnourished dams had approximately the same extent of reduction in body-weight, mandibular weight and long-bone weight.
3. The malnourished group had more cells in the mandible although cell size was the same as that of controls.
4. In contrast, in the long bones, the malnourished group had fewer cells than did controls whereas cell size was unchanged.
5. Calcium content was the same in long bones of both groups, but was less in the mandibles of pups from malnourished dams. Ca metabolism as measured by ^{45}Ca uptake was unchanged in the long bones, but was increased in the mandibles of the malnourished group shortly after birth. Calcification patterns at birth in these bones correlated well with alkaline phosphatase (EC 3.1.3.1) activity.
6. These findings indicate that the mandibles and long bones of offspring are affected differently by protein-energy malnutrition during the mother's gestation. Prenatal nutritional stress resulted in a disturbance of the pituitary-adrenal system. Increased adrenal corticosterone could possibly be related to the different observed changes in bone metabolism.

Protein deprivation has been shown not only to reduce the weights of the various organs of the offspring, but also to decrease cell number and size (Zeman, 1970; Endo *et al.* 1974; Van Marthens & Shimomaye, 1978). Little attention, however, has been paid to the prenatal effects of malnutrition on hard tissue, although in postnatal studies newborn rats that were subjected to protein-energy malnourishment had reduced growth and development of hard tissues such as bones (DiOrio *et al.* 1973; Shrader & Zeman, 1973; Nakamoto & Miller, 1977) and tooth germs (Fabian *et al.* 1972; Nakamoto *et al.* 1979). Thus, we studied how maternal protein deficiency affects the growth of mandibles and long bones and whether biochemical changes occur in these bones. We also determined the adrenal corticosterone content, which has been known to play some role in the modification of the organic phase of bone formation (Vaughan, 1975; Cohen *et al.* 1977).

MATERIALS AND METHODS

Eighteen pregnant Sprague-Dawley rats were given a standard stock diet until day 13 of gestation. Nine control dams were given 250 g protein/kg diet from the 13th day of gestation until birth. The diet of the nine malnourished rats was isoenergetic, but provided only 60 g protein/kg (Table 1). Within 8 h of delivery, all pups, both male and female, were combined and weighed, since no weight difference is attributable to sex in early ages (Nakamoto & Miller, 1977). Then, the same number of pups from each dam in respective groups were killed by cutting the carotid artery.

The left and right mandibles of each pup were split in the middle with a sharp knife and removed. The tooth germ of molars and incisors, soft tissue, and mandibular nerves were carefully removed from the mandibular body and discarded. The distal part of the femur,

Table 1. *Composition of diets (g/kg)*

Group...	Control	Malnourished
Dietary protein (casein)	250	60
Glucose	172	267
Sucrose	168	172
Dextrin	172	262
Maize oil (Mazola®) (ml)	150	150
Mineral mix*	40	40
Choline chloride, 500 g/l (ml)	4	4
Cellulose	35	35
Vitamin mix†	10	10
Methionine	—	1.0

* Rogers-Harper mineral mix (Rogers & Harper, 1965; Teklad Test diets, Madison, WI).

† AIN vitamin mixture 76 (ICN Pharmaceuticals, Inc., Cleveland, OH). Composition of vitamin mix (g/kg): thiamin hydrochloride 0.6, riboflavin 0.6, pyridoxine hydrochloride 0.7, niacin 3.0, calcium pantothenate 1.6, folic acid 0.2, biotin 0.02, vitamin B₁₂ (0.1% trituration in mannitol) 1.0, dry retinyl palmitate (275.2 mg/g) 0.8, cholecalciferol trituration (10 mg/g), 0.25, dry α -tocopheryl acetate (500 mg/g) 10.0, menadione 0.005, sucrose (finely powdered) 981.225.

the knee joint, and the proximal part of the tibia were removed and cleaned of soft tissue. The distal part of the femur and the proximal part of the tibia included cartilaginous epiphysis and a portion of the developing trabeculae of metaphysis. Cleanliness of the mandibular body and long bone was checked under a magnifying glass before the bones were weighed.

Six randomly-selected right and left mandibles and four long bones were pooled to provide a large enough sample to complete the analyses for protein, DNA and RNA. Bone samples were prepared as previously described (Nakamoto & Miller, 1977). Protein was determined by the method of Lowry *et al.* (1951) and bovine serum albumin was used as a standard. DNA and RNA were determined by the method of Prasad *et al.* (1972). Furthermore, the alkaline phosphatase (*EC* 3.1.3.1) and acid phosphatase (*EC* 3.1.3.2) activities were measured as previously described (Nakamoto & Miller, 1979*b*). Since alkaline and acid phosphatase activities have diurnal changes, pups used in the determination of these enzyme activities were killed between 13.00 and 15.00 hours. Adrenals of pups were removed and weighed. Corticosterone content was measured according to the method described by Porter *et al.* (1977).

A group of pups from control and malnourished groups were injected with ⁴⁵Ca (specific activity 22.5 mCi/mg; New England Nuclear, Boston, MA) at a dose of 1 mCi/kg. Pups were killed 0.5–1 h after injection to determine Ca uptake (Mallek *et al.* 1979; Nakamoto *et al.* 1979; Nakamoto & Miller, 1979*a*). The Ca content of these samples was measured by atomic absorption spectrophotometry (Model 280 atomsorb; Fisher Scientific Co., Fairlawn, NJ). ⁴⁵Ca contents of the bone were then measured using a liquid-scintillation spectrometer (Beckman Model LS-3145T, Irvine, CA). Efficiencies were determined by external channels ratio. Values were analysed by Student's *t* test (Dixson & Massey, 1969).

RESULTS

The average daily food intake was 23(SE 3)g for pregnant dams on the 25 g protein/kg diet and 15(SE 4)g for those dams on the 60 g protein/kg diet. On delivery, total body-weights of pups of the malnourished group were 20% less than those of the controls (Table 2). The weights of the mandibles and long bones in the malnourished group were 19 and 20% less respectively than those of the controls (Table 2).

Table 2. Average weights of body, mandible and long bone
(Mean values with their standard deviations for nine dams; three pups per dam)

Group	Dietary protein (g/kg)	Total body-weight (g)	Mandible weight† (mg)	Long bone weight† (mg)
Control	250	6.92	18.11	14.03
SD		0.26	2.31	0.77
Malnourished	60	5.53***	14.72***	11.21***
SD		0.43	0.95	1.01

† Six randomly-selected mandibles and four randomly-selected long bones were combined respectively and then divided mathematically to determine the average weight of bone.

*** $P < 0.001$.

Table 3. Protein, DNA and RNA contents, alkaline (EC 3.1.3.1) and acid (EC 3.1.3.2) phosphatase activities, calcium content and ^{45}Ca uptake, adrenal weight and corticosterone content in mandibles (M) and long bones (L) of newborn rats

Group	Dietary protein g/kg	Protein† (mg)		DNA† (μg)		RNA† (μg)		Alkaline phosphatase‡		Acid phosphatase‡	
		M	L	M	L	M	L	M	L	M	L
Control	250	33.73	55.93	12.5	168.2	118.5	328.4	30.31	10.64	4.72	2.74
SD		4.02	7.11	3.3	12.4	30.1	54.1	5.70	3.24	1.04	0.25
Malnourished	60	44.31***	59.05	18.5***	135.0***	142.5	331.5	52.81***	10.00	5.33	2.33
SD		3.61	7.53	2.4	15.1	43.5	36.4	15.82	3.21	1.61	0.39

Group	Dietary protein g/kg	Ca content (mg)		Ca uptake ($\times 10^4$)		Adrenal weight§ (mg)	Corticosterone§ ($\mu\text{g}/100$ mg tissue)
		M	L	M	L		
Control	250	130.6	68.2	22.3	10.0	13.41	1.86
SD		44.4	15.1	4.2	5.6	1.50	0.44
Malnourished	60	66.7***	69.6	40.9**	14.6	8.22***	2.39*
SD		12.0	12.6	13.7	6.3	1.50	0.67

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† Mean values for protein, DNA and RNA contents expressed per g tissue within 8 h of birth. Each average mandible and long bone value represents a measurement from nine dams (three pups per dam). All calculations are based on wet weight.

‡ Alkaline and acid phosphatase activities are expressed as Sigma units/mg protein as described previously (Nakamoto & Miller, 1979b). Each mandible group mean represents an average of nine dams (with one pup per dam) in both control and malnourished groups. Each long bone group mean represents an average of six dams (two pups per dam) in the control and malnourished group.

|| Calcium content expressed per g tissue at the time of birth. Ca uptake expressed as disintegrations/min per mg Ca. Each mandible group mean represents an average of nine dams (one pup per dam) in the control and malnourished groups. Each long bone group mean represents an average of six dams (two pups per dam) in the control and malnourished groups.

§ The weight represents the pool of six adrenals; average of five pools. Corticosterone content represents an average of five dams (three pups per dam).

Mandibular DNA and protein contents per g tissue were higher in the malnourished group than in the controls (Table 3) but the protein content per mandible did not differ. The RNA content of the mandibles was the same in both groups. The DNA content of the long bones of the malnourished groups was less. Protein and RNA contents of long bones were not different when expressed per g tissue; however, when the values were expressed per long bone the measurements were less in the malnourished group ($1.53(\text{SE } 0.29)\mu\text{g}$) than in controls ($2.33(\text{SE } 0.25)\mu\text{g}$).

Alkaline phosphatase activity in the mandibles of the malnourished group was higher than in the controls but, in the long bones, it did not differ. Acid phosphatase activity in both the mandibles and long bones did not differ between groups (Table 3).

Ca content in mandibles was less in the malnourished group, but ^{45}Ca uptake was greater. Ca content and ^{45}Ca uptake in the long bones was similar in both groups (Table 3).

Adrenal weights in the malnourished group were lower than those of the controls, but the corticosterone content was greater in the malnourished group than in the controls (Table 3).

DISCUSSION

Although the effects of protein deprivation during pregnancy have been studied in various organs (Zeman, 1970; Endo *et al.* 1974; Van Marthens & Shimomaye, 1978), little attention has been paid to bone metabolism. Thus, we selected the mandible and long bone as examples of membranous and endochondral bones respectively (Weinmann & Sicher, 1955).

Because rats given the protein-deficient diet ate less food than did the controls, they experienced an energy deficiency as well as a protein deficiency. One can argue that, as a result of the slight decrease in food intake in the malnourished mothers, the fetal bone metabolism may have been affected to a certain extent by other nutrients such as Ca or vitamin D or both, which, in addition to protein and energy, play a role in hard-tissue metabolism. Theoretical calculations of nutrients such as vitamin D and Ca in the diets of the protein-deficient group, however, showed that these nutrients were not deficient simply because the food intake was lower in this group. Malnourished mothers more than sufficiently met basic nutritional requirements ((US) National Academy of Science, 1972).

Body-weight and the weights of mandibles and long bones of pups in the malnourished group were all reduced to approximately the same extent at the time of birth. This finding suggests that the prenatal effects on the mandible caused by the deficiency of protein and energy are different from a similar nutritional stress applied in the early postnatal period where mandibles and long bones are differently affected (Nakamoto & Miller, 1977; Nakamoto & Miller, 1979 *a, b*). The fact that the critical growth period of the mandible is earlier than that of the long bone (Nakamoto & Miller, 1977) may partly explain why the weights of the mandibles and total body-weights were similarly reduced.

Bone cell number (DNA) in the mandible was greater in the malnourished group, regardless of whether the mean values (with SE) were expressed as either per mandible (250 g protein/kg, $0.225(0.023)\mu\text{g}$; 60 g protein/kg, $0.275(0.018)\mu\text{g}$) or g tissue (Table 3). This increased cell number was unexpected; in other organs such as the brain, kidney, liver and heart, decreased cell number is reportedly decreased during prenatal protein malnutrition (Zeman, 1970; Younoszai *et al.* 1978). The cell size (protein:DNA) calculated by the standard method (Enesco & Leblond, 1962; Winick & Noble, 1965) was not different between groups. RNA:DNA, an index of cellular activity, was slightly lower in the malnourished group.

The long bone cell number, however, was decreased in the malnourished group, but cell sizes and cellular activity (RNA:DNA) did not differ between the groups. The general differences between the mandibles and long bones observed in the present study may be

due partly to a regional difference in developmental pattern in these bones (Weinmann & Sicher, 1955).

Alkaline phosphatase activity is closely related to the calcification of bones (Salomon, 1974). Higher alkaline phosphatase activity in the mandibles in the malnourished group explains why Ca uptake was increased in this group at the time of killing. The increase in alkaline phosphatase activity, which reflects bone calcification, would imply that Ca content in the mandibles of malnourished animals should be greater, although it was in fact lower. Because these measurements were made immediately after birth, these apparent discrepancies in the mandible reflect differences in Ca metabolism occurring in the postnatal animals as opposed to the Ca metabolism *in utero*. This reasoning is further supported by the values in long bone. In long bones, no differences between malnourished and control group were found in alkaline phosphatase, Ca content, or ^{45}Ca uptake. Hormonal changes that occur in newborns on birth have been discussed (Pitkin, 1975). The discrepancy in Ca content and ^{45}Ca uptake in mandibles may also possibly be a function of a postnatal compensatory response to reduction in calcification during the early prenatal period.

Another way to express bone calcification indirectly is by the Ca:DNA value. This value was less in the mandibles from the malnourished group and could possibly account for the decrease in weight of the mandibles. On the other hand, increased ^{45}Ca uptake suggests that Ca in the apatite crystal may not be related to the condition of live bone cell activity immediately after birth.

In the long bones, Ca content did not differ between groups, suggesting that the decreased weight of long bones may be partly due to the decrease of organic matrix. In fact, the long bones in the malnourished group showed decreased amounts of protein on a per long bone basis. Calcification per cell in the long bone was not different.

It could be disputed that not all the cells in bones are involved in the calcification process, and for this reason the expression of Ca:DNA may not be valid in a quantitative sense. Relative comparison between control and malnourished groups, however, does provide some idea of the basic differences between groups.

Acid phosphatase activity is known to be associated with bone resorption (Marks, 1974). Acid phosphatase activities in both mandibles and long bones were not different between groups, suggesting that the rate of catabolism in these bones is the same.

Corticosterone reportedly affects the organic phases of bones (Vaughan, 1975; Cohen *et al.* 1977) and is elevated in protein-deficient animals (Lunn *et al.* 1976) and humans (Olusi *et al.* 1977). Increased adrenal corticosterone content in the malnourished group compared with the controls suggests that this prenatal stress resulted in a disturbance of the pituitary-adrenal system. This concomitant change in pituitary-adrenal function and subsequent increase of adrenal corticosterone content may be related to the different changes in bone metabolism observed in the present study.

In an *in vitro* study, Dietrich *et al.* (1979) found increased collagen synthesis in the fetal rat calvaria incubated with corticosterone. Thus, increased adrenal corticosterone content in our study may be partly responsible for a higher protein content per g mandibular tissue. Although one might argue that the long bone should be affected as well, differential effects of glucocorticoid on other organ systems (Clark & Vigals, 1979) as well as fetal bones (Mosier *et al.* 1981) have been well documented. Thus, the evidence presented here shows that the prenatal period is extremely important for healthy growth and development of bones, and the mandible and long bone in newborns are differently affected by prenatal malnutrition.

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