Influence of High Protein Diets on Cartilage and Bone Formation in Rats[1]

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ABSTRACT The influence of diets containing combinations of high protein and low calcium on discrete stages of bone formation was investigated in 28-day-old rats. A bone matrix-induced bone forming system was utilized to determine the stages of endochondral ossification that were being affected. Mesenchymal cell proliferation as assessed by [^{3}H]-thymidine incorporation and ornithine decarboxylase activity were unchanged in animals fed a high protein (80% casein)/normal calcium (0.61% Ca; 0.40% P) diet. However, osteogenesis was reduced by 78% in the rats fed high protein/normal calcium as measured by ^{45}Ca incorporation. Alkaline and acid phosphatase activities in bone were increased 2.5 and 2.3 times, respectively, reflecting increased matrix turnover induced by the high protein availability. Bone that did form was not remodeled nor was there evidence of marrow formation. The animals were normocalcemic and normophosphatemic and showed no evidence of acidosis. A combination diet of high protein and low calcium resulted in a 62% reduction of cell proliferation and chondrogenesis and a 98% inhibition of bone formation. High dietary protein-induced osteoporosis in animals is due to a failure of osteogenesis at the stage of ossification possibly a result of restricted availability of calcium at the site of mineralization. J. Nutr. *111:* 804–816, 1981.

INDEXING KEY WORDS protein · calcium · bone formation

Osteoporosis has been demonstrated in experimental animals fed a high protein diet (1–3). In view of the high incidence of osteoporosis in America and the fact that the American diet is relatively high in protein, it has been hypothesized that this type of diet may, in part, be responsible for the high occurrence of this bone disease (4–7). It has been well established that increased consumption of protein in humans and animals results in increased urinary calcium excretion (5, 8–13). However, the underlying mechanisms by which high protein diets adversely affect bone are unknown.

Discrete stages of bone formation in the epiphyseal growth plate are difficult to evaluate, and their analysis is rather complex due to spatial and temporal problems. However, the use of a demineralized bone matrix-induced endochondral bone forming system eliminates these problems and permits the study of discrete synchronous phases of bone development (14–17). As previously described (15), 3 days after matrix implantation mesenchymal cell proliferation occurs, which is followed by cartilage differentiation on day 7, calcification on day 10 and eventually ossification and bone marrow development on days 14–21. It should therefore be possible to determine at which stage

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TABLE 1

Diet compositions

Component	Diet			
	N-Ptn/N-Ca	H-Ptn/N-Ca	N-Ptn/L-Ca	H-Ptn/L-Ca
	g/kg	*g/kg*	*g/kg*	*g/kg*
Casein[1]	200	800	200	800
D,L-Methionine	3	3	3	3
Cornstarch	150	12	150	12
Sucrose	500	0	500	0
Alphacel[2]	50	63	50	63
Corn oil	50	63	50	63
AIN Mineral Mix[3]	35	44	0	0
AIN Vitamin Mix[4]	10	13	10	13
Choline bitartrate	2	3	2	3
AIN Mineral Mix without calcium	0	0	35	44

[1] 95.0%, minimum dry milk protein; 1.5%, maximum, butterfat, 2.2% ash; 0.033% Ca; 0.123% P. [2] Non-nutritive bulk: 0% protein; 5 ppm heavy metals; 78% crude fiber (90% β 1 $\rightarrow$ 4 Glucan); 200 ppm Ca; trace, P. [3] (g/kg): $CaHPO_4$, 500.0; NaCl, 74.0; $HOC(COOK)(CH_4COOK)_4 \cdot H_2O$, 220; K_2SO_4, 52.0; MgO, 24.0; manganous carbonate (43–48% Mn), 3.5; ferric citrate (16–17% Fe), 6.0 zinc carbonate (70% ZnO), 1.6; cupric carbonate (53–55% Cu), 0.3; potassium iodate (KIO_3), 0.01; $Na_2SeO_3 \cdot 5H_2O$, 0.01; $CrK(SO_4)_2 \cdot 12H_2O$, 0.55; sucrose, finely powdered, 118.0. [4] (per kg mixture): thiamin HCl, 600 mg; riboflavin, 600 mg; pyridoxine HCl, 700 mg; nicotinic acid, 3 mg; D-calcium pantothenate, 1.6 mg; folic acid, 200 mg; vitamin B-12, 1 mg; vitamin A, 800 mg; DL-α-tocopheryl acetate, 20 g; cholecalciferol, 2.5 mg; vitamin K, 5.0 mg; sucrose 972.9 g.

of endochondral bone formation various diet regimes exert their influence.

This investigation compares the influence of high protein/normal calcium, normal protein/low calcium and high protein/low calcium diets on endochondral bone differentiation. High protein ingestion resulted in a specific reduction of osteogenesis without affecting cell proliferation or chrondrogenesis.

MATERIALS AND METHODS

Experimental animals and diets

Male rats of Long-Evans strain (age 21–22 days, weight 50–75 g) were randomized into four groups fed diets of: normal protein, normal calcium (N-Ptn/N-Ca) high protein, normal calcium (H-Ptn/N-Ca); normal protein, low calcium (N-Ptn/L-Ca); or high protein, low calcium (H-Ptn/L-Ca). The composition of these diets is shown in table 1. The pelleted diet was assayed for calcium by atomic absorption analysis and for phosphorus (18). The calcium (Ca^{++}) and phosphorus (P_i) contents of the diet were 0.49 and 0.35% for normal protein/normal calcium, 0.61 and 0.40% for high protein/normal calcium, 0.02 and 0.01% for normal protein/low calcium and 0.03 and 0.015% for high protein/low calcium.

Animals were pair fed throughout the experiment to ensure that all groups ate the same amount of food. Rats were placed on the experimental diets for 2 weeks before matrix implantation. There were four rats per group per experiment and each experiment was repeated at least two times. Tap water was provided ad libitum except in one experiment when an additional group of rats fed the high protein/normal calcium was given water enriched for calcium (10 mg/liter, $CaCl_2$) ad libitum. All animals were kept at 22 ± 2° with a 12-hour light/dark cycle. Animals were weighed daily.

Preparation of matrix and implantation

Demineralized bone matrix prepared from rat diaphyses was implanted subcutaneously (14–17) into rats 2 weeks after being placed on the experimental diets. The day of implantation was designated as day 0, and all surgical procedures

and autopsies were performed between 1000 and 1100 hours. The present study investigated four distinct stages of matrix-induced endochondral bone formation by explanting the decalcified bone matrix (plaques) at various time intervals (14): *1*) proliferation of mesenchymal cells on *day 3*, monitored by [^{3}H]thymidine incorporation and ornithine decarboxylase activity; 2) chondrogenesis on *day 7*, monitored by $^{35}SO_4$ incorporation into proteoglycans; 3) cartilage calcification on *day 9*, as indicated by alkaline phosphatase and ^{45}Ca incorporation; and *4*) osteogenesis on *days 11, 14* and *21*, also monitored by ^{45}Ca incorporation. The activities of alkaline and acid phosphatases were also determined because of their close association with bone formation and resorption, respectively. Two plaques (implants) from each rat were assayed in duplicate, and there were four rats in each group. The data reported are an average of three separate experiments.

Ornithine decarboxylase (EC 4.1.17; ODC) assay

Day 3 plaques were dissected out, homogenized (Polytron homogenizer, Brinkman, Westbury, NY) in ice-cold buffer containing 50 mM tris-HCl, 5 mM dithiothreitol and 1 mM EDTA at pH 7.4 and centrifuged (Sorvall RC-5B, Norwalk, CT) at 30,000 × *g* for 30 minutes. The supernatant was assayed as described by Jänne and Williams-Ashman (19) and Rath and Reddi (20). ODC activity was expressed in picomoles as $^{14}CO_2$ released from L-[1-^{14}C]ornithine (56.6 mCi/mmole) per hour per milligram protein. Protein was determined according to Lowry et al. (21) with bovine serum albumin as standard.

[^{3}*H*]*thymidine incorporation*

Methyl-[^{3}H]thymidine (6.7 Ci/mmole) was injected intraperitoneally at a dose of 1 μCi/g body weight in 0.15 M NaCl (14). Two hours after injection, rats were bled by cardiac puncture and then killed by CO_2 asphyxiation. The subcutaneous button-like plaques were removed, weighed and homogenized in ice-cold 10% (wt/vol) trichloroacetic acid (TCA) containing 1 mM thymidine (nonradioactive). The tubes were kept cold for 30 minutes and subsequently centrifuged at 4,500 × *g* for 15 minutes. The supernatant was saved for determination of acid-soluble radioactivity by liquid-scintillation spectrometry, and the precipitate was washed twice with ice-cold 10% (wt/vol) TCA without thymidine. The washed precipitate was hydrolyzed in 2 ml 10% TCA at 90° for 20 minutes and then immediately placed in an ice bath for 30 minutes. Samples were then centrifuged for 15 minutes at 4,500 × *g*, and the radioactivity in the aliquots of the supernatants was determined. The deoxyribonucleic acid (DNA) content of the hydrolyzed supernatant was determined by the diphenylamine procedure (22). The results were expressed as: micrograms DNA per milligram tissue; counts per minute per microgram DNA in acid-insoluble precipitate; and counts per minute per milligram tissue in the acid-soluble supernatant and acid-insoluble precipitate.

[^{35}S] *incorporation into proteoglycans*

Sodium [^{35}S]sulfate (950 mCi/mmole) was injected intraperitoneally in 0.15 M NaCl at a dose of 1 μCi/g body weight. Two hours after injection, the plaques were dissected out, weighed and homogenized on ice-cold 20% (wt/vol) TCA with 20 mM sodium sulfate. The tubes were kept cold for 30 minutes and then centrifuged at 4,500 × *g* for 15 minutes. An aliquot of the supernatant was saved to determine the radioactivity in the acid-soluble component, whereas the precipitate was washed twice in ice-cold 20% TCA without sodium sulfate. The precipitate was hydrolized in 2 ml 10% TCA (wt/vol) for 10 minutes at 90°, placed in an ice-water bath for 30 minutes and centrifuged; the supernatant was assayed for DNA as described above. Radioactivity in the supernatant was determined. The sediment after acid hydrolysis was then dissolved in 23 M formic acid for 10 minutes at 90°. The combined radioactivity from the TCA hydrolysate and formic

TABLE 2

Blood chemistries

Diet	pH ($n = 4$)	BUN. ($n = 8$)	Alkaline phosphatase ($n = 16$)	Ca ($n = 16$)	P ($n = 16$)
		mg/100 ml	*U/μl*	*mM/liter*	*mM/liter*
N-Ptn/N-Ca	7.43	16 ± 1	18.9 ± 1.8	1.50 ± 0.10	6.33 ± 0.20
H-Ptn/N-Ca	7.47	40 ± 3[a]	7.7 ± 1.1[a]	1.35 ± 0.05	6.37 ± 0.25
N-Ptn/L-Ca	7.41	22 ± 2	7.0 ± 1.2[a]	0.62 ± 0.10[a]	5.75 ± 1.10
H-Ptn/L-Ca	7.45	32 ± 3[a]	9.6 ± 1.2[a]	0.93 ± 0.07[a]	5.78 ± 0.28

[a] Significant at $P \leq 0.001$.

acid digest was reported as counts per minute per microgram DNA and represented $^{35}SO_4$ incorporation into acid-precipitable proteoglycans.

Alkaline and acid phosphatases and ^{45}Ca incorporation

$^{45}CaCl_2$ (15–20 Ci/g) at a dose of 1 μCi/g body weight was injected intraperitoneally in 0.15 M NaCl. The plaques were dissected 2 hours later, weighed, homogenized in ice-cold 0.15 M NaCl and 0.003 M $NaHCO_3$ (pH 7.4) and centrifuged at 20,000 × g for 15 minutes at 4°. The supernatants were assayed for alkaline and acid phosphatase using *p*-nitrophenol phosphate (Sigma Chemical Co., St. Louis, MO), as a substrate with 0.1 M barbital buffer (pH 9.3) for alkaline phosphatase and a 0.1 M acetate buffer (pH 5.0) for acid phosphatase as described previously (14, 15). Soluble protein was also assayed in the supernatant (19). The radioactivity of the supernatant was counted and referred to as the salt soluble pool of ^{45}Ca available to the tissue.

The sediment was stirred with 0.1 M $CaCl_2$ in 0.005 M tris-HCl (pH 7.5) for 30 minutes, centrifuged and washed twice with 0.005 M tris-HCl (pH 7.5). The washed sediment was then stirred in 0.5 M HCl for 16 hours and centrifuged, and aliquots were taken for determination of radioactivity and for determination of calcium by atomic absorption spectrometry. The ^{45}Ca uptake data was used to measure the rate of calcification during the pulse of label, and total calcium was a monitor of total calcification (23). A detailed description of this method for concurrent determination of phosphatases and ^{45}Ca incorporation will be published elsewhere (unpublished observations).

Blood chemistry

At the time of autopsy, blood was collected and serum analyzed for calcium by atomic absorption analysis, phosphorous (18), alkaline phosphatase as described above, and blood urea nitrogen (Bun-Tel Clinical Assay Kit, Pfizer Scientific, New York). Blood gas analysis was performed on heparinzed blood drawn in a siliconized glass syringe from a left ventricle puncture. The animal was anesthesized with ether prior to exsanguation. A PHM72-MK2 Digital Acid-Base Analyzer with BMS 2 MK2 Blood Micro System (Radiometer, Copenhagen) was used.

Histological observations

Plaques at various stages of development were fixed in Bouin's fixative, embedded in paraffin sections and were stained with hematoxylin and eosin.

Statistical analysis

Linear regression analyses were performed on body weight data with computation of the regression coefficient ($b_{x.y}$) at the 95% limits of confidence (L_1, L_2). All other data was analysed with the Student's *t*-test and means ± SE are reported.

TABLE 3

Average growth per day and regression coefficients for growth curves

Diet	Growth	$b_{y.x}$[1]	L_1, L_2[2]
	g/day		
N-Ptn/N-Ca	2.1	2.35	2.12, 2.59
H-Ptn/N-Ca	2.7[a]	3.70[a]	3.52, 3.89
N-Ptn/L-Ca	2.0	2.46	2.08, 2.83
H-Ptn/L-Ca	1.2[a]	1.32[a]	1.19, 1.46

[1] Regression coefficient. [2] 95% confidence levels. [a] Significant at $P \leq 0.001$.

RESULTS

Serum chemistry

Analyses of blood from rats on the experimental diets are shown in table 2. All rats had normal blood gas profiles and were normophosphatemic. Rats fed a high protein/normal calcium diet were normocalcemic. The plasma alkaline phosphatase was reduced by 59% and the blood urea nitrogen (BUN) was increased 2.5 times compared to rats fed a normal protein/normal calcium diet (control group). The groups fed either a normal protein/low calcium or a high protein/low calcium diet were markedly hypocalcemic and also had a reduction in alkaline phosphatase. Rats fed a high protein/low calcium diet also exhibited an elevated BUN. While the high protein supplement in the diet resulted in decreased alkaline phosphatase and a high BUN value and while low calcium also resulted in a decreased alkaline phosphatase but no change in the BUN, when these two variables were combined in one diet the result was a slight increase in alkaline phosphatase and a slight decrease in BUN

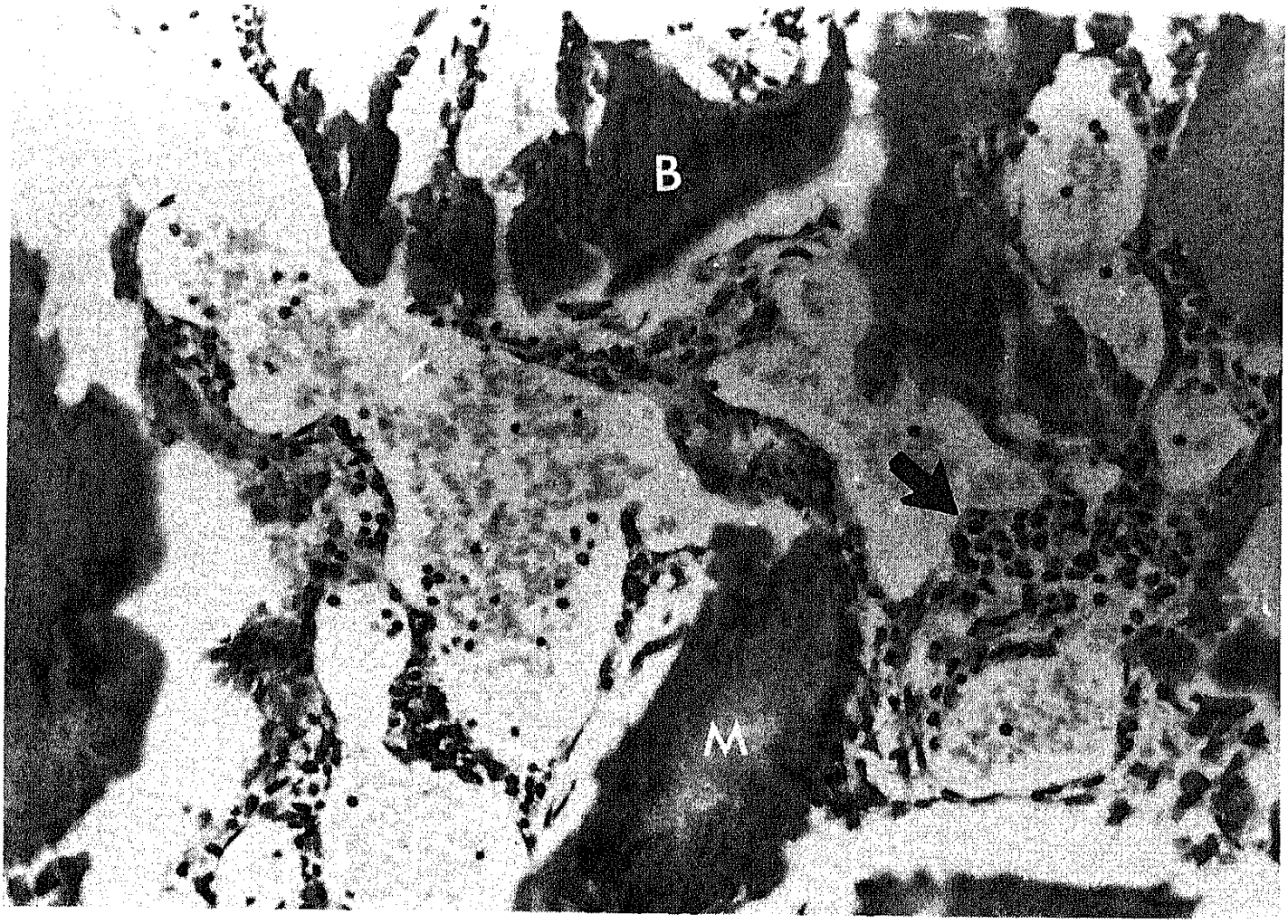

Fig. 1 Day 14 implanted matrix of rat fed normal protein/normal calcium diet. M, implanted matrix; B, bone; arrow, bone marrow. 150×.

(only 49% of control) compared to rats fed a high protein/normal calcium diet. Therefore while high protein diets induce uremia and lower alkaline phosphatase activity, the absence of calcium in the diet resulted in a reduction in the degree of uremia.

Body weights

Young rats fed a high protein/low calcium diet exhibited the least amount of weight gained in the 21 days of observation (table 3). While initially the growth curve was similar to the control, by day 10 the amount of weight gained was markedly reduced. Rats fed a high protein/normal calcium diet initially showed a stimulated rate of growth which eventually slowed down to that of the control group (normal protein/normal calcium). The group fed normal protein/low calcium was similar to the control group.

Linear regression analysis was performed on the growth curves for all animals studied and the regression coefficient calculated is shown in table 3. The rate of growth is proportional to the $b_{x.y}$ value. This table illustrates that the average rate of growth for the time examined is much faster in the animals fed a high protein/normal calcium diet and much slower in the animals fed high protein/low calcium.

Histology

The histological changes observed in response to implantation of demineralized bone matrix have been described in detail (17). Briefly, in control rats on *day* 3, there was extensive proliferation of mesenchymal cells. On *day* 7, chondrogenesis was maximal with a characteristic cartilage

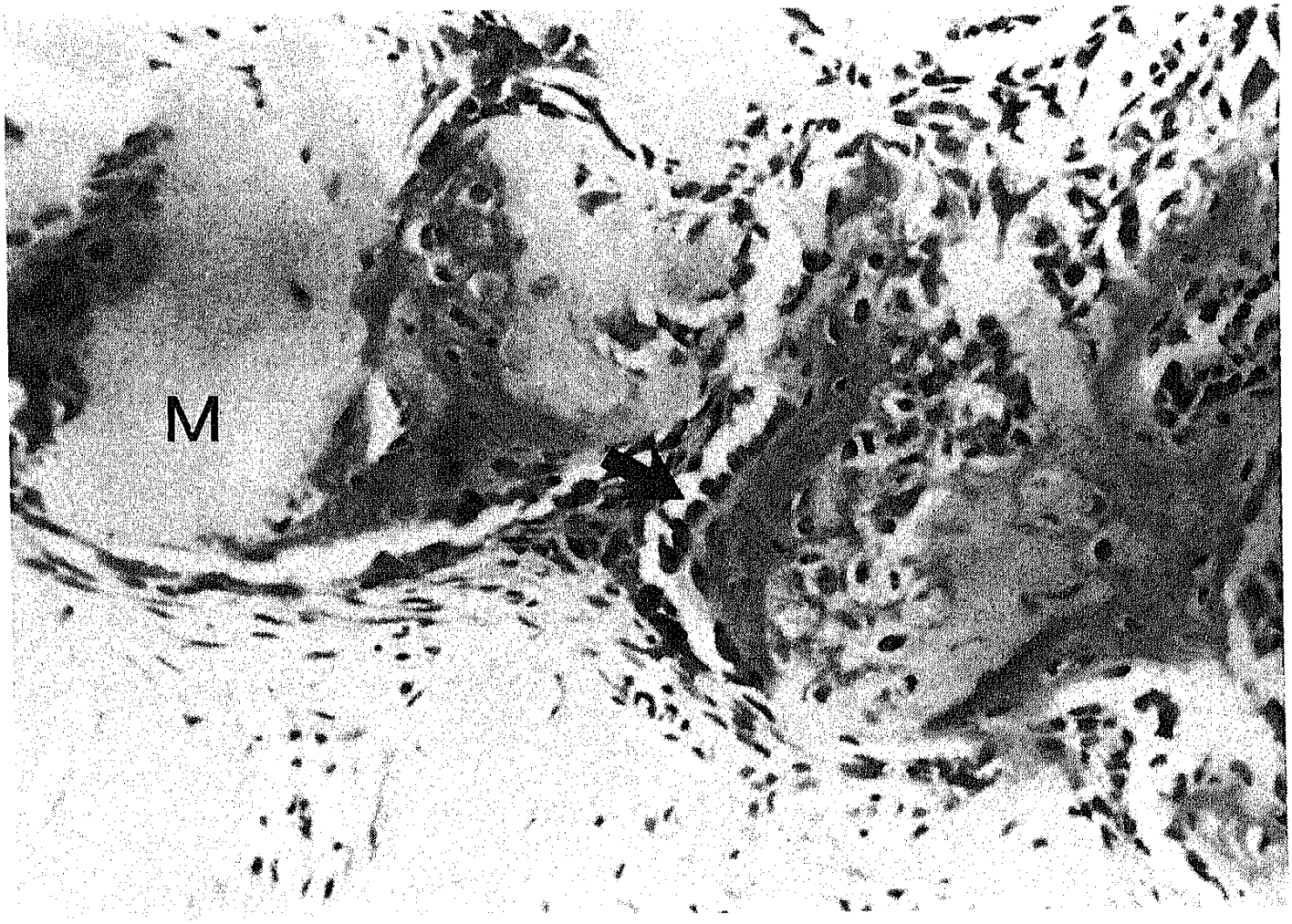

Fig. 2 Day 14 implanted matrix of rat fed normal protein/low calcium. M, matrix; osteoblasts, arrow. 150×.

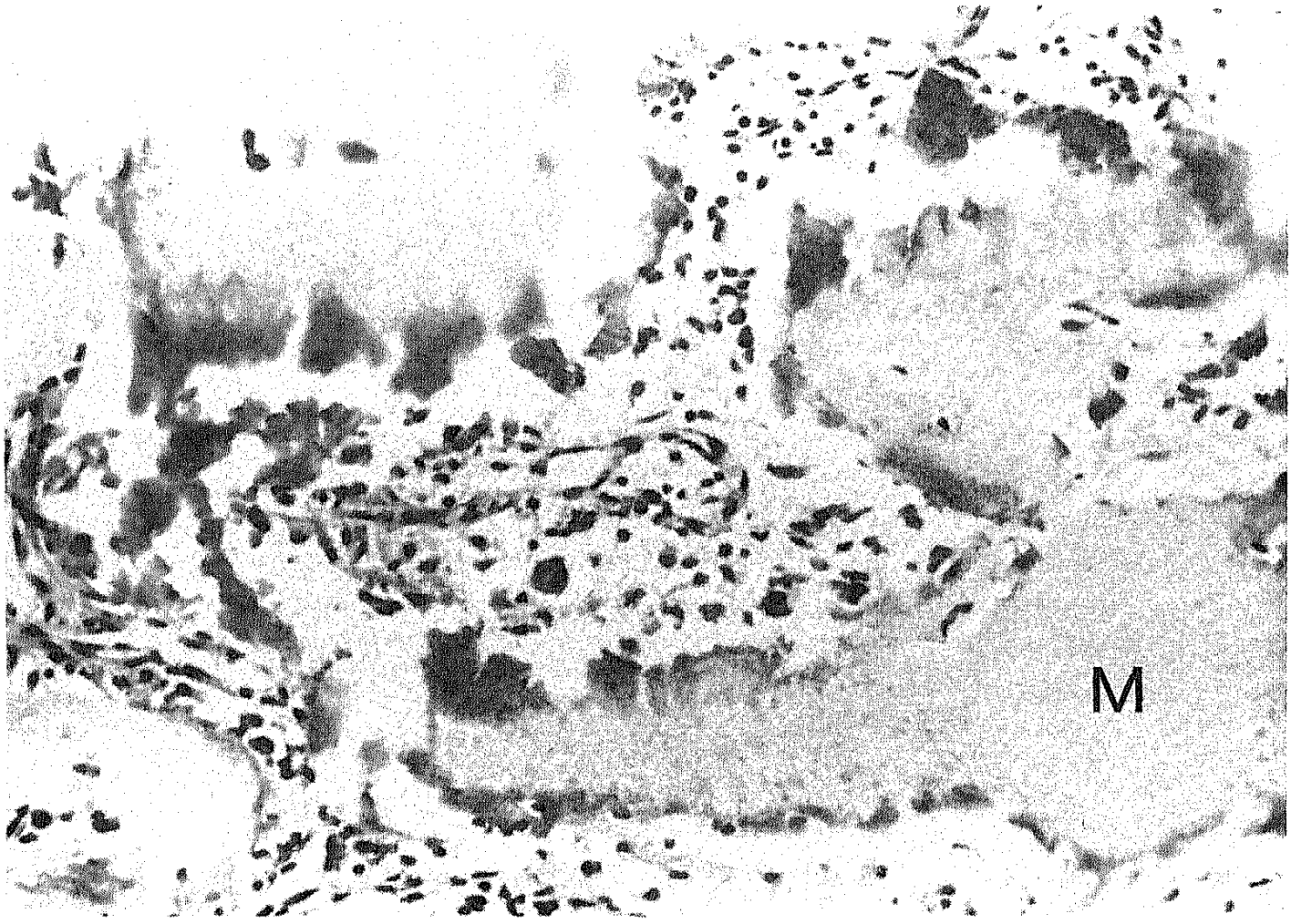

Fig. 3 Day 14 implanted matrix of rat fed high protein/normal calcium. Note the absence of marrow. M, matrix. 150×.

matrix. On *day 9*, vascular invasion was evident with several chonodrolytic foci. Maximal osteogenesis and remodeling were observed on *day 14*. Hematopoietic bone marrow differentiation was maximal in the newly formed ossicle on *day 21* (16).

In rats fed a high protein/low calcium diet the sequential cellular transitions appeared to be retarded. On *day 3*, the implanted matrix was surrounded by mesenchymal cells as in the rats fed the control diet (normal protein/normal calcium). On *day 7*, in comparison to control rats, there were scanty areas of chondrogenesis. On *day 14*, where one observed histologic evidence of osteogenesis in control rats (normal protein/normal calcium) [fig. 1], the animals on the high protein/low calcium diet exhibited only cartilage (fig. 4). There were no striking differences among the other groups on *days 3* and *7*, however on *day 14* the amount of bone observed in developing plaque tissues of the animals fed a high protein/normal calcium diet was markedly reduced compared to the controls. The bone that was evident in the group fed the high protein/normal calcium diet was not remodeled and there was no evidence of hematopoiesis (fig. 3). Bone formation that did occur appeared similar to the control in those animals fed a normal protein/low calcium diet (fig. 2).

Therefore, rats fed a high protein diet in the presence of normal amounts of calcium exhibited: a) decreased bone formation; and b) failure of marrow cavity formation. These results are further substantiated by the physiological and biochemical parameters to be documented below.

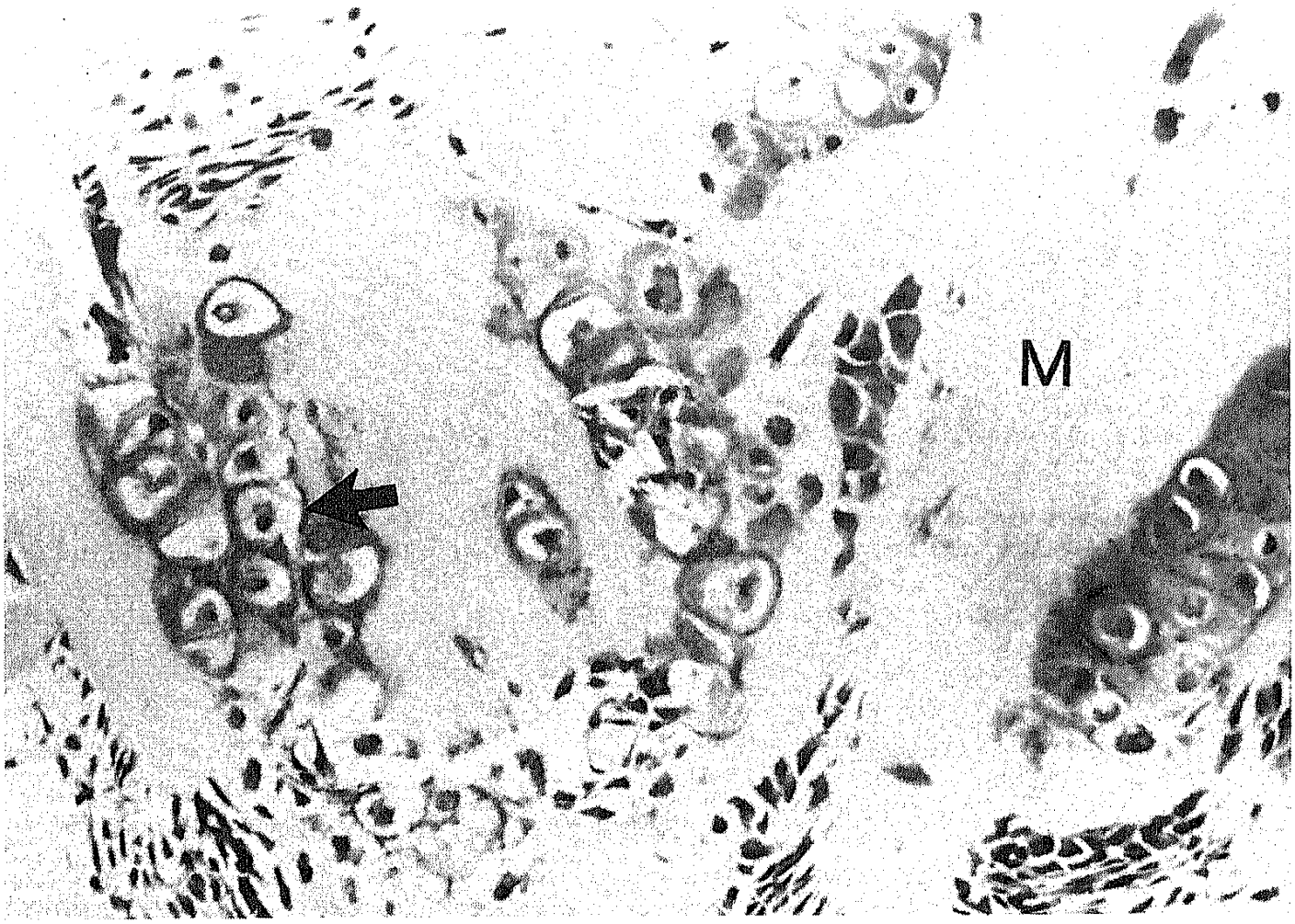

Fig. 4 Day 14 implanted matrix of rat fed high protein/low calcium. Note the absence of bone formation. Only chondrocytes (arrow) are present. M, matrix. 150×.

Mesenchymal cell proliferation (day 3)

Ornithine-decarboxylase (ODC) activity in plaque tissues from rats fed a high protein/normal calcium diet 3 days after matrix implantation was 53% of control values (table 4). Animals on the high protein/low calcium regime had ODC activities only 38% that of the control. Rats fed a normal protein/low calcium diet were not significantly different from the control animals for any of the parameters measured. The rate of thymidine incorporation was similar for all groups except those on the high protein/low calcium diet and these rats also had a predicted decrease in total DNA content of the tissue. Therefore the degree of cell proliferation was markedly impaired in those animals fed a combination diet of high protein/low calcium, whereas low calcium alone was without effect and high protein alone only slightly reduced the thymidine incorporation (table 4).

Chondrogenesis (day 7)

The influence of the experimental diets on incorporation of $^{35}SO_4$ into proteoglycans is shown in table 5. Chondrogenesis was markedly impaired in animals given the high protein/low calcium diet, analyzed by activity per cell or activity per tissue. The other diet regimes were without effect on cartilage formation.

Osteogenesis (day 14)

The influence of the high protein diets on osteogenesis as measured by calcification and phosphatase activities is shown in tables 6 and 7, respectively. Animals

TABLE 4

Influence of high protein diets on cell proliferation [ornithine decarboxylase (ODC) activity and [^3H]-thymidine incorporation (^3HtdR)]

Diet (n = 8)	ODC	^{3}HtdR	DNA
	pmol $^{14}CO_2$ released/ mg protein/hour	*cpm/mg tissue*	*μg/mg tissue*
N-Ptn/N-Ca	395 ± 74	365.2 ± 60	1.00 ± 0.12
H-Ptn/N-Ca	210 ± 70[a]	256.1 ± 62	0.79 ± 0.09
N-Ptn/L-Ca	268 ± 38	274.7 ± 14	0.92 ± 0.16
H-Ptn/L-Ca	150 ± 8[b]	231.3 ± 17[b]	0.66 ± 0.16[a]

[a] Significant at $P < 0.10$. [b] Significant at $P < 0.01$.

fed high protein/normal calcium, normal protein/low calcium and high protein/low calcium exhibited an inhibition in the rate of ^{45}Ca incorporation and total calcium accumulation in the developing tissue. Animals fed the normal protein/low calcium diet exhibited the most severely reduced osteogenesis with a 98 and 96% reduction in ^{45}Ca incorporation and total calcium accumulated, respectively. While the high protein diet alone only resulted in a 60% reduction of ^{45}Ca incorporation, the high protein/low calcium combination resulted in a 79% reduction. Therefore the presence of excess protein in a diet deficient in calcium results in a lesser decrease (79 versus 98%) in bone formation. The addition of calcium-enriched drinking water to the regimen of those animals on the high protein/normal calcium diet did not stimulate the ^{45}Ca incorporation to normal levels, although there was a fourfold increase in the total calcium incorporated into the tissue compared to the rats on the same diet without calcium-enriched drinking water.

The ^{45}Ca activity in the plasma was consistent for all groups. The salt soluble pool of ^{45}Ca, representing the extracellular calcium which would be available for mineralization, was significantly reduced in rats fed the high protein/low calcium diet, but unaffected in the other regimes.

The influence of high protein diets on alkaline and acid phosphatases in the developing tissues is shown in table 7. Rats fed the high protein/normal calcium diet exhibited a significantly higher activity of alkaline and acid phosphatases in the developing plaque. Whereas those animals on a normal protein/low calcium diet exhibited a marked reduction in alkaline phosphatase activity. The animals fed the high protein/low calcium diet exhibited an increase in the alkaline phosphatase. Therefore while a low calcium diet tends to decrease alkaline phosphatase and a high protein diet causes an increase, the combination of low calcium and high protein results in an increased activity. The addition of calcium-enriched drinking water to those animals on the high protein/normal calcium diet was without effect on the alkaline phosphatase, but did reduce the acid phosphatase value to normal levels compared to those animals on the same dietary regime without the enriched drinking water. While high dietary protein results in a reduction of calcification, it stimulates alkaline phosphatase in the tissue.

Similar results were obtained looking at the same parameters in the metaphyses

TABLE 5

Influence of high protein diets on $^{35}SO_4$ incorporation into protelglycan in day 7 plaque tissues[1]

Diet	$^{35}SO_4$	$^{35}SO_4$
	cpm/μg DNA	*cpm/mg tissue*
N-Ptn/N-Ca	18.10 ± 2.8	99.9 ± 23
H-Ptn/N-Ca	16.53 ± 3.3	105.5 ± 12
N-Ptn/L-Ca	17.61 ± 2.5	122.2 ± 4
H-Ptn/L-Ca	11.52 ± 1.39[a]	77.2 ± 12[a]

[1] Results expressed as means ± SE. [a] Significant at $P \leq 0.001$.

TABLE 6

Influence of high protein diets on calcium-45 incorporation into day 14 plaque tissues[1]

Diet	^{45}Ca-salt soluble pool	^{45}Ca	Ca	Plasma ^{45}Ca
	cpm/mg tissue	*cpm/mg tissue*	*μmole/mg tissue*	*cpm/10 μl*
N-Ptn/N-Ca	7,534 ± 730	3,324 ± 206	62.0 ± 2.8	5,470 ± 88
H-Ptn/N-Ca	7,635 ± 980	1,314 ± 220[a]	5.4 ± 1.7[a]	6,221 ± 89
N-Ptn/L-Ca	6,260 ± 830	60 ± 14[a]	2.36 ± 0.18[a]	5,781 ± 305
H-Ptn/L-Ca	2,573 ± 660[a]	711 ± 17[a]	6.40 ± 0.24[a]	7,850 ± 250
H-Ptn/N-Ca + Ca-enriched drinking water[2]	7,642 ± 820	1,346 ± 180[a]	20.8 ± 0.17[a]	5,334 ± 179

[1] Results expressed as means ± SE. [2] 10 mg/liter $CaCl_2$. [a] Significant at $P < 0.01$.

of these animals (table 8). A high protein/normal calcium diet resulted in a reduction of ^{45}Ca incorporation and an increase in alkaline phosphatase. Low calcium diets with normal protein resulted in reduced alkaline phosphatase. In rats fed the low calcium diet, the rate of incorporation of ^{45}Ca was not reduced (table 8). The combination of high protein/low calcium resulted in an increased alkaline phosphatase activity and a severely inhibited rate of ^{45}Ca incorporation in the metaphyses similar to the developing plaque tissue.

DISCUSSION

The influence of diets containing combinations of high protein and low calcium on discrete stages of bone formation has been investigated. As a result of high dietary protein intake, we have observed decreased bone formation as measured by the rate of ^{45}Ca incorporation and decreased total calcium accumulated in the developing matrix-induced osseous tissue.

While other workers have documented osteoporosis in animals fed a high protein diet (1–3), we have shown that the inhibition of endochondral bone formation seen in rats fed a high protein/normal calcium diet is due to a specific failure of osteoid formation and subsequent mineralization, and not to mesenchymal cell proliferation or chondrogenesis which appeared normal. In a study of Alaskan Eskimos, the decreased bone density was ascribed to the acidic effect of the high meat diet (24). However, in this study acute metabolic acidosis can be ruled out as a possible cause for failure of bone formation as blood gases and pH values were normal. Ingestion of high protein has been known to result in calciuria (5, 8–13). As also reported here, previous workers have found that even though the rats were hypercalciuric, they were normocalcemic which speaks against a defect in calcium absorption by the gut. Also, with the ingestion of high protein it has been shown that intestinal calcium-binding protein activity is unaffected (25). It is generally believed that the cause of the calciuria is decreased tubular resorption of calcium (7, 13). Possibly the calciuria may also be, in part, due to the shunting of fecal calcium excretion to the urine (10). Recently, Whitney and Draper (26) have shown that the degree of calciuria was directly related to the sulfur amino acid content of the diet. Nevertheless, whatever the cause of the calciuria is, the animals are still absorbing normal amounts of calcium and are normocalcemic.

Young growing rats fed a high protein/normal calcium diet showed increased levels of alkaline phosphatase in the developing tissue even though they did not ossify. The increase in tissue levels may be related to a compensatory increase associated with a failure of bone formation. This has also been observed with low phosphate diet-induced rickets (27).[2] However, low levels of serum alkaline phosphatase were observed in rats fed high protein. By analogy, hy-

[2] Reddi, A. H. & Binderman, I. Manuscript in preparation.

pophosphatasia of unknown etiology is characterized by radiological and histological features resembling rickets (28). Such patients usually exhibit low plasma alkaline phosphatase. However, similar to our observations for high protein diets the tissue levels of alkaline phosphatase are normal (29–31). Despite the discovery of alkaline phosphatase in skeletal tissue over 50 years ago (32, 33) little is known concerning its role in bone formation (26, 34). Perhaps high protein diets result in alkaline phosphatase being made by osteoblasts, which is either defective or not being released extracellularly, hence causing a defect in bone formation.

Acid phosphatases, on the other hand, have been implicated in bone remodeling (27) and this enzyme was found to increase during bone remodeling in normal developing matrix-induced endochondral bone (13, 14, 16). Rats fed a high protein diet had an increased tissue acid phosphatase which would reflect increased matrix turnover induced by the high protein availability. The high enzyme activity may also reflect a compensatory increase due to lack of bone remodeling and marrow formation as observed histologically.

High phosphorous diets are known to induce osteoporosis which may be attributed to a secondary hyperparathyroidism induced by a slight depression of serum calcium associated with increased circulating phosphate (35–38). Casein (800 g/kg), the protein source in the experimental diets, is a phosphoprotein with 0.123% P. In the high protein diets the casein only contributed an additional 0.1% of total weight as P. This value is below the 1.8% P necessary to induce osteoporosis (35–38). Furthermore the animals were normophosphatemic. These observations would rule out the possibility that failure to form bone in high protein diets is a result of increased phosphorous absorption.

TABLE 7

Influence of high protein diets on alkaline and acid phosphatase (ϕ) activities in day 14 plaque tissues

Diet	Alk ϕ	Acid ϕ
N-Ptn/N-Ca	19.24 ± 1.77	29.55 ± 4.2
H-Ptn/N-Ca	37.01 ± 5.03[a]	68.81 ± 5.8[a]
N-Ptn/L-Ca	6.21 ± 3.0[a]	29.7 ± 1.7
H-Ptn/L-Ca	39.40 ± 4.45[a]	28.26 ± 4.6
H-Ptn/N-Ca + Ca-enriched drinking water[1]	39.40 ± 4.45[a]	35.82 ± 5

[a] Significant at $P < 0.01$. [1] 10 mg/liter $CaCl_2$.

TABLE 8

Influence of high protein diets on calcium incorporation and phosphatase activities into metaphyses

Diet	^{45}Ca	Alkaline phosphatase
	cpm/mg tissue	*μ/mg tissue*
N-Ptn/N-Ca	29,860 ± 552	127 ± 20
H-Ptn/N-Ca	8,760 ± 452[a]	200 ± 15[a]
N-Ptn/L-Ca	24,160 ± 211	65 ± 15[a]
H-Ptn/L-Ca	6,115 ± 200[a]	186 ± 47

[a] Significant at $P < 0.001$.

A low calcium diet with normal protein intake resulted in hypocalcemia and reduction in bone formation. In this case the reduced bone formation could be explained by the decreased availability of calcium. However, the small amount of bone that did form had normal marrow associated with it. The tissue alkaline phosphatase activity in these rats was severely reduced, reflecting how levels of calcium modulate the induction of the enzymatic activity. A combination diet of high protein and low calcium proved to be detrimental in an additive fashion, in that bone did not form as examined histologically and biochemically for the 22 days of observation. Similar to the calciuric response described by Margen (11), the addition of excess calcium to a diet high in protein was without effect on bone formation.

Bell and co-workers (39) report no effect of high protein diets on bone *resorption* in rats. This correlates well with the data presented here as we report a lack of bone remodeling and a failure of bone *formation*. Since mesenchymal cell proliferation and cartilage formation seem normal, then high dietary protein-induced

osteoporosis may be due to a specific defect which restricts the availability of calcium at the site of mineralization.

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