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Effects of dietary protein and calcium on the skeleton of undernourished young rats*)

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Children suffering from kwashiorkor show signs of osteopenia (1) and delayed ossification status (2). However, the "kwashiorkor child" does not only have a low protein intake, but there is also a dietary shortage of calcium, vitamin D and other nutrients, and also a restricted energy supply.

The findings reported in various studies on the effect of a low-protein intake on skeletal development and bone composition in experimental animals are conflicting. The observation that the skeleton of the growing and the adult rat fed a low-protein diet becomes osteoporotic (3) has not been confirmed (4, 5). Weaned rats fed a low-calcium diet for a period have been found to develop signs of rickets, followed later by osteoporosis without any alterations in the diet (6).

The purpose of the present investigation was to examine the effect of varying the levels of dietary protein and calcium on the skeleton of young rats.

Material and methods

The study was performed on 60 male, 3-week-old Sprague-Dawley rats. They were housed in groups of 5 in plastic cages. The room was artificially lit for 12 hours a day, the temperature was kept at 21–23 °C and the relative humidity at 50–60 per cent. After delivery from the farm, the rats were acclimatized to laboratory conditions by feeding them for 4 days a standard rat-cube diet containing 24.1 per cent protein, 1.16 per cent calcium and 1.00 per cent phosphorus. The rats were then stratified according to body weight and randomized into 6 experimental groups each of 10 rats. The mean body weight at the time they were fed the diet was 65.1 g (S.D. 2.4 g).

The period of the study was 5 weeks. Each group of animals was fed one of the diets shown in table 1.

The protein contents of the diets were 5, 15 and 40 per cent, referred to below as the low, moderate and high levels. The calcium contents were 0.12 and 0.45 per cent – designed low and normal, respectively. The phosphorus content was kept constant at 0.3 per cent. The protein source

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was casein and variations in the protein level were provided by replacing part of the casein by maize starch. DL-methionine was added in proportion to the protein content –0.1, 0.3 and 0.8 per cent respectively.

Pelleted food and water were given *ad libitum.* The body weight and food consumption were recorded weekly. Water consumption was recorded for a period of 2 weeks. The measurements of the variables were performed on 5 animals from each group. After 5 weeks all the rats were killed by exsanguination under ether anesthesia.

The serum levels of calcium, phosphorus and albumin were determined spectrophotometrically (7, 8, 9) and magnesium was assayed by atomic absorption spectrophotometry (10).

The *bone length* of the tibias and femurs was measured with a caliper. The *wet weight* of the bones was determined after soaking them in distilled water for 24 hours. The *total volume* of the bone was determined by water displacement. The *dry weight* of the femur was measured after defatting in several changes of acetone and alcohol, and then air-dried at 70 °C for 36 hours. The *bone ash weight* was obtained after ashing at 600 °C for 14 hours. The *external diameter* of the femur midshaft was measured on an antero-posterior radiograph with a micrometric scale lens. The *width of the epiphyseal cartilage* of proximal tibia was measured on decalcified 5 μ thick haematoxylin-eosin-stained histological sections. The epiphysis was projected on a sheet of paper with a camera lucida mounted on a microscope, and the width of the epiphyseal cartilage was measured at a magnification of ×100.

Microradiographic examination

Undecalcified specimens of the femur were embedded in methylmethacrylate and cut into slices about 100 μ thick with an annular saw machine[1]). One longitudinal 100 μ thick section from the proximal part of the femur, including the head, and one transverse 100-μ section from the femur midshaft were microradiographed. The microradiographs were quantified with an image analyser (Quantimet 720 equipped with an image editor)[2]) connected to a television camera mounted on a microscope.

The total bone area, the *area between the internal and external circumference,* the *total area of the transverse section,* and the *lengths of the external and internal circumference* of the bone were measured on the transverse section. The mean width of cortex was calculated according to the formula

$$\textit{mean width} = 2 \times \frac{\text{area between outer and inner perimeter}}{\text{length of outer and inner perimeter}}$$

The *total area* and the *bone area of the femoral* head, and the *circumference* and area of the *bone trabeculae* were measured on the proximal part of the femur. As an index of the thickness of the trabeculae the ratio between area and perimeter was calculated.

Bone mass was expressed in terms of the dry weight, and *bone density* as the dry weight per unit of volume. Analogously, *bone mineral mass* was taken as the ash weight, and *bone mineral density* as the ash weight per unit of volume.

[1]) Microslice 2. Metals Research Ltd, Melbourn, Herts, U.K.

[2]) Imanco, Cambridge, U.K.

Table 1. Percentage composition of the test diets.

Diet	Pr 5/Ca	Pr 5/–Ca	Pr 15/Ca	Pr 40/Ca	Pr 40/–Ca
Casein	5.0	5.0	15.0	40.0	40.0
Maize starch	59.8	60.05	49.8	24.4	25.2
Glucose	15.0	15.0	15.0	15.0	15.0
Soya oil	12.5	12.5	12.5	12.5	12.5
Cellulose	4.0	4.0	4.0	4.0	4.0
DL-Methionine	0.1	0.1	0.3	0.8	0.8
Choline chloride	0.4	0.4	0.4	0.4	0.4
Vitamin mixture[a])	1.0	1.0	1.0	1.0	1.0
Salt mixture[b])	0.5	0.5	0.5	0.5	0.5
$CaCO_3$	0.55	0.65	0.65	1.00	0.1
$CaHPO_4$ H_2O	1.15	–	0.85	0.4	–
Na_2HPO_4	–	1.20	–	–	0.4

Composition of the vitamin mixture; milligram per kilogram of diet

[a]) The vitamin mixture provided in mg/kg diet: Thiamine 4, riboflavin 12, niacin 40, pyridoxine 5, biotin 0.3, Ca-panthothenate 10, folic acid 0.5, p-aminobenzoic acid 250, vitamin B_{12} 0.02, inositol 30, vitamin A 12,000 I.U., vitamin E 40, vitamin D_3 1760 I.U., vitamin K_3 1.5, choline chloride 100, ascorbic acid 50.

[b]) Composition of the salt mixture; milligrams per kilogram of diet: Mg 150, Mn 53, Fe 66, Zn 80, Cu 12.5, Co 0.6, and I 1.1.

Statistical methods

1. One-way analysis of variance between groups having different protein but normal calcium content of the diet.
2. Two-way analysis of variance with protein and calcium contents of the diet as independent variables.
3. Student's t-test for examining the differences between the groups having different calcium but the same protein content of the diets.

The 1 % level of significance was chosen (12).

Results

During the 5-weeks period of the study the animals in all groups gained very little in weight – between 6 and 10 g. The food intake was very low – from 5.4 to 7.3 g/d (table 2). Despite their restricted growth, the animals seemed to be in good health, no signs of disease being observed during the experiment. However, 3 animals on the medium protein/low calcium diet (group Pr15/-Ca) died during the third and fourth week. Cannibalism altered the protein intake of the survivors of this group, which was therefore excluded from the study.

Body weight and food consumption did not differ significantly between the groups during the experiment. The water consumption showed great difference between the experimental groups. The animals fed a high-protein diet consumed twice as much water as the animals fed the medium or the low protein diets ($P < 0.01$), and those fed a low calcium diet consumed less water than those fed a normal calcium diet (table 2). The serum value of calcium showed a small but significant decrease in the rats on a normal calcium diet when the protein content of the diet was

Table 2. Final body weight. Food, protein and water consumption per day.

Diet group		Body weight g	Food g	Protein g	Water ml
Pr 5/Ca	Mean	75.0	7.3	0.36	21.8
	S.D.	0.8	0.1		0.8
Pr 5/–Ca	Mean	76.3	6.1	0.31	9.5
	S.D.	0.8	0.1		0.3
Pr 15/Ca	Mean	76.3	5.7	0.85	22.0
	S.D.	0.8	0.0		0.0
Pr 40/Ca	Mean	71.0	5.8	2.32	37.5
	S.D.	1.6	0.2		0.2
Pr 40/–Ca	Mean	76.1	5.4	2.16	32.9
	S.D.	1.9	0.2		0.3

reduced (table 3). Furthermore, reduction of the calcium content in the diet from normal to low gave a significant decrease in serum calcium in the animals on the high protein but not those on the low protein diets (table 3).

Serum phosphorus and magnesium did not differ between the experimental groups, and serum albumin was only reduced significantly in the low-protein/low-calcium groups compared with the rats on the other diets (table 3).

Bone parameters

The length, external diameter, and volume of femur and tibia of the 5 diet groups did not differ significantly. Bone mass and bone mineral mass

Table 3. Serum Ca, P, Mg, and Albumin mM/l.

Diet group		Ca	P	Mg	Albumin
Pr 5/Ca	Mean	2.40[a])	1.93	1.49	37.7
	S.D.	0.11	0.44	0.24	1.5
Pr 5/–Ca	Mean	2.35	2.31	0.91	27.5[c])
	S.D.	0.23	0.16	0.12	3.2
Pr 15/Ca	Mean	2.54	2.57	1.00	39.8
	S.D.	0.10	0.12	0.18	2.6
Pr 40/Ca	Mean	2.68[b])	2.73	1.32	36.0
	S.D.	0.12	0.47	0.26	3.5
Pr 40/–Ca	Mean	2.38	2.89	1.35	39.4
	S.D.	0.05	0.48	0.20	0.5

Statistical analysis

Serum Ca:

[a]) Pr 5/Ca < Pr 15/Ca or Pr 40/Ca ($P < 0.01$)

[b]) Pr 40/Ca > Pr 40/–Ca ($P < 0.01$)

Serum albumin:

[c]) Pr 5/–Ca < Pr 5/Ca or Pr 15/Ca or Pr 40/Ca or Pr 40/–Ca ($P < 0.01$)

Table 4. Recorded values of the bone variables for the femur.

Diet Group		Length cm	Volume cm^3	Dry weight g	Ash weight g	Dry wt/volume g/cm^3	Ash wt/volume g/cm^3	In/Or
Pr 5/Ca	Mean	2.62	0.2400	0.1469	0.0917	0.612	0.382	1.662
	S.D.	0.06	0.0171	0.0114	0.0080	0.018	0.015	0.058
Pr 5/–Ca	Mean	2.54	0.2333	0.1137	0.0674	0.487	0.289	1.455
	S.D.	0.08	0.0161	0.0085	0.0052	0.019	0.012	0.033
Pr 15/Ca	Mean	2.67	0.2532	0.1543	0.0950	0.609	0.375	1.605
	S.D.	0.05	0.0122	0.0073	0.0052	0.007	0.008	0.052
Pr 40/Ca	Mean	2.59	0.2350	0.1576	0.1008	0.671	0.429	1.777
	S.D.	0.06	0.0112	0.0113	0.0077	0.035	0.028	0.091
Pr 40/–Ca	Mean	2.59	0.2397	0.1219	0.0722	0.524	0.308	1.427
	S.D.	0.07	0.0150	0.0089	0.0056	0.030	0.014	0.062
Effect of:				Statistical analysis				
Protein		n.s.	n.s.	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	n.s.
Calcium		n.s.	n.s.	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	n.s.
Interaction								$P < 0.01$

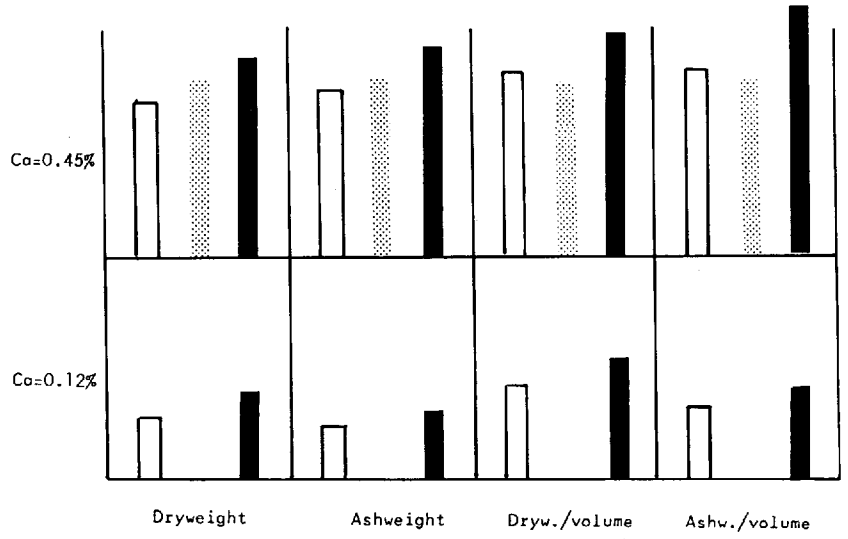

Fig. 1. Dry weight, ash weight, dry weight/volume and ashweight/volume in relation to the animals on a diet with 15 per cent protein and 0.45 per cent calcium. White columns 5, grey columns 15 and black columns 40 per cent protein.

were positively significantly correlated with protein intake but independent of the calcium level (table 4). Bone density and bone mineral density was significantly increased in the high-protein groups (table 4). Irrespective of the protein level of the diet, the bone mass and density and the bone-mineral mass and density were significantly lower for the animals on a low than those on a normal calcium diet (table 4).

The protein and calcium level of the diet interacted on the composition of the bone when expressed as the ratio between inorganic and organic matter of the bone. The animals on a high protein/normal calcium diet had the highest and those on a high protein/low calcium diet the lowest In/Or ratio (table 4). (Fig. 1).

At a normal calcium diet a high protein intake increased the bone area, the percentage bone area and the mean trabecular width of the femoral head significantly. At both low and high protein intakes the mean width of cortex measured midshaft on the femur was evidently not associated with the protein intake. In the groups with a low calcium diet the mean width of cortex was significantly depressed both at a high and a low protein intake, and there were no significant differences in the total transversal area of femur (table 5).

The epiphyseal cartilage plate was significantly wider in rats on the low protein/normal calcium diet than in those with higher protein intakes. The width of the epiphyseal plate was evidently unaffected by the calcium intakes (table 5). There was no histological evidence of osteomalacia.

Table 5. Values of the bone variables for the femur from microradiographs and the proximal epiphysis of the tibia.

		Transverse section of femur (mid-shaft)				Femoral head		Tibia
		Bone area mm^2	Area of section mm^2	Mean width of cortex mm	Bone area mm^2	% bone of head area	Mean width of trabecule	Epiphyseal cartilage of prox. tibia mm
Diet group								
Pr 5/Ca	Mean	1.898	4.329	0.300	1.864	17.5	0.038	0.181
	S.D.	0.163	0.333	0.017	0.334	3.5	0.009	0.025
Pr 5/–Ca	Mean	1.834	4.925	0.260	2.149	21.1	0.036	0.165
	S.D.	0.096	0.321	0.018	0.115	4.2	0.006	0.036
Pr 15/Ca	Mean	1.854	4.545	0.288	2.485	20.0	0.038	0.145
	S.D.	0.139	0.267	0.018	0.762	4.6	0.088	0.018
Pr 40/Ca	Mean	1.962	4.374	0.304	3.536	31.7	0.056	0.134
	S.D.	0.109	0.270	0.019	0.472	2.2	0.003	0.010
Pr 40/–Ca	Mean	1.666	4.349	0.249	1.894	16.4	0.030	0.148
	S.D.	0.102	0.221	0.016	0.450	3.1	0.007	0.014
				Statistical analysis				
Effect of protein		n.s.	n.s.	n.s.	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
Effect of calcium								
5 % protein		n.s.	n.s.	$P < 0.01$	n.s.	n.s.	n.s.	n.s.
40 % protein		$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$	n.s.

Discussion

Reliability of the experimental model

In animals fed a standard laboratory ration a positive correlation has been found between age, body weight and the weight and density of the bone (13, 14). In studies of the bone variables in the rat and man, adequate normalization for body size is necessary for a reliable interpretation of bone mass data (15). In studies of skeletal variables in animals fed diets of different compositions both age and body weight are therefore factors of paramount importance.

To overcome the tendency for animals on a low protein diet to eat less (16, 17), various techniques have been used, such as force feeding, pair feeding and meal feeding (5, 18, 19, 20). As these feeding methods are not physiological, they may introduce a distorting factor. When a rat, which is a nibbler by nature, is forced to become a meal eater, alterations in food adsorption and changes in the composition of the body develop (21, 22). There is also evidence that the nitrogen balance is dependent on the number of meals taken a day (23). Even the time of day when the meal is taken may, it is claimed, influence the utilization of the food (24). In the present study all the animals were fed ad libitum. Nonetheless, the rats' consumption was low, though almost identical for all the diet groups, as also was their gain in weight. By normal standards for food intake and growth pattern all the groups were clearly undernourished, but in all other respects they met the criteria for reliable inter-group comparisons.

The causes of undernourishment

The observation that a diet low in protein or with an imbalanced amino acid composition impairs the appetite and retards weight gain has been ascribed to the influence on the appetite centre exerted by the amino acid pattern in the brain (25).

In order to ensure that all the diets used in the present study contained the same amount of amino acids, they were supplemented with methionine in proportion to be protein content. The effect of the added methionine on the food intake and body growth was examined separately in a short-term experiment. Young rats were fed the high-protein diet used in this experiment and were compared given the same diet without addition of methionine. The control animals consumed significantly more and grew better, and the undernourishment of the rats in the present study could therefore be attributed to the methionine added to the test diets. It has been reported that diets containing 2.5 and 4.5 per cent of methionine can lead to extensive alterations in the organs of young rats and also inhibition of bone growth, which was identical in degree to that seen in the weight-control animals, on a basilar diet, and these changes were thus considered to be nonspecific (26).

Analyses showed that approximately one third of the added DL-methionine was oxidized to the sulphoxide. Methionine sulphoxide has not been found to exert any toxic effects on young rats (27). Since the largest amount of methionine present in the test diets used in the present study was only 0.8 per cent, it was judged highly improbable that the methionine added to the diet produced specific bone alterations.

Bone alterations in relation to protein intake

Two forms of osteoporosis have been postulated, namely, matrix osteoporosis – that is, decreased deposition of bone matrix resulting from an abnormally low protein intake – and mineral osteoporosis, in which there is normal deposition of bone matrix but enhanced destruction of old bone to meet the calcium requirement arising from the intake of diets adequate in protein but deficient in calcium (24). Experiments on young and adult rats have shown that either protein or calcium deficiency will give severe osteoporosis (28). However, it has also been reported that monkeys fed a low-protein diet (18) and young rats submitted to protein or energy restriction (19) did not develop changes in bone composition.

In the present study a low protein intake was associated with a small, though significant, reduction in bone mass. On the other hand, the rats with a high protein/normal calcium diet showed a significantly elevated bone density. The marked effect on bone tissue induced by a low protein intake reported by El-Maraghi et al. was probable due to the difference in the weight of the experimental and the control animals. When both pair-fed and weight-matched control rats were used, thus to some extent avoiding the implications of a difference in weight of the compared animals, it has been found that a low protein intake led to a reduction in bone mass (5). The results of the present study are consistent with this experience. However, the conclusion drawn from Shenolikar's study that animals on a low-protein diet have an increased percentage ash of the bone is not borne out by the observations of either the present or the referred studies (15). It seems reasonable to conclude that protein deficiency in young animals leads to a reduction in bone mass. The evidence for an effect on bone density is inconclusive.

Restriction of the intake of a normal diet will result in a higher bone density in growing animals compared with free access to the same diet (29, 30). The present results suggest that low intake of a high-protein diet may constitute a greater restriction than the intake of the same amount of a low-protein diet. Support for this is found in lower height of the growth plate observed in the high compared to the low-protein diet groups.

Bone alterations in relation to both protein and calcium intake

Variations in protein intake may affect the skeleton either by direct influence on the synthesis of the bone matrix, or indirectly through changes in the calcium metabolism. It has been reported that a high protein intake increases calcium absorption (31, 28); moreover, protein-deprived young rats have shown a lower calcium absorption and lower serum calcium levels than normal controls (4). On the other hand, it has been reported that a high protein intake exerts a pronounced calciuric effect in young men, leading to a negative calcium balance (32, 33) and that rats fed a high-protein diet display increased absorption and increased excretion of calcium, but no loss of skeletal calcium (34). Among the rats of the present study with a normal calcium intake the serum calcium level was significantly lower for those fed the lower than those fed the high-protein diet. From these observations it would thus appear that the effect of a high protein intake on calcium absorption is not to meet the calcium

requirements for the skeletal metabolism but rather to compensate for the increased excretion of endogenous calcium. Thus if the calcium content of the diet is low and the percentage absorption is already high, the increase in calcium absorption due to a high protein intake may not compensate for the increased excretion. This would account for the absence of a correlation between serum calcium and protein intake in the present study when the calcium content of the diet was as low as 0.12 per cent. It may also explain the difference in the effect of protein on the bone composition at low and normal calcium intakes.

Adaption to a low calcium intake

In the present study a low-calcium diet combined with either a high- or a low-protein diet was associated with a significantly depressed bone-mineral mass and bone density. At a low calcium intake there was increased endosteal bone resorption but normal periosteal growth compared to normal calcium intake. These observations on the effect of a low calcium intake on the skeleton of the young rat confirm those reported on adult rats (35, 36).

Reduction in the protein content of the diet was associated with less trabeculae bone, and reduction in the calcium content of the diet reduced the cortical bone. This suggests that the effect of protein and calcium on the composition of bone is governed by different mechanisms.

Summary

A study has been made of the effects of various levels of dietary protein and calcium on the skeleton of young undernourished rats. The data for the study were obtained from physical properties of bone, from microradiographs of various parts of femur and histological sections of the bone.

There were significant associations between a low protein intake and low bone mass, irrespective of calcium intake, and between a high protein intake and a high bone density at a normal calcium content of the diet. The influence of dietary protein on the composition of the bone was dependent on the calcium intake. Reduction in the protein level was associated with less trabecular bone and reduction in the calcium reduced mainly the amount of cortical bone.

Zusammenfassung

Es wurden Untersuchungen über die Wirkung von verschiedenen Protein- und Kalziummengen in der Diät auf das Skelett von jungen unterernährten Ratten durchgeführt.

Die Ergebnisse bezogen sich auf die physischen Eigenschaften der Knochen und auf mikroradiographische Untersuchungen verschiedener Teile der Oberschenkelknochen und auf Knochengewebeschnitte.

Es zeigten sich deutliche Beziehungen zwischen Niedrig-Protein-Diät und geringer Knochenmasse, unabhängig von der Kalziumeinnahme, wie auch zwischen Hoch-Protein-Diät und hoher Knochendichte bei normalem Kalziumgehalt in der Diät. Der Einfluß von Diätprotein auf die Knochenzusammensetzung war von der Kalziumeinnahme abhängig. Eine Herabsetzung der Proteinmenge wurde mit weniger spongiösem Knochengewebe in Verbindung gebracht, eine Herabsetzung der Kalziummenge verminderte hauptsächlich die Menge von kortikalem Knochengewebe.

Key words: bone – protein deficiency – calorie restriction – calcium deficiency

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