BONE METABOLISM AND COMPOSITION IN THE PROTEIN-DEPRIVED RAT

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SUMMARY

1. Bone and calcium metabolism in protein-deprived 5-week-old male rats who exhibited no biochemical or histological features of rickets were compared with age- and weight-matched control animals.

2. Intestinal calcium absorption was significantly lower in protein-deprived rats. This was associated with a smaller exchangeable calcium pool and lower bone formation as measured by calcium accretion and proline incorporation into hydroxyproline. However, calcium resorption from bone barely changed in this group and a disproportionately high fraction of the calcium pool continued to be excreted.

3. As a consequence, retarded bone growth occurred in the protein-deprived rat, leading to bones which were shorter and lighter than those of age-matched control animals, the epiphyseal regions appearing to bear the brunt of the growth retardation. This was not accompanied by a change in quality of bone, as the percentage ash content of extirpated bone remained normal.

4. Though alternative hypotheses are possible, the data are consistent with a primary disturbance of intestinal, and possibly renal tubular, calcium transport, perhaps associated with impaired synthesis of calcium-binding protein. An independent depression of collagen synthesis is likely.

Key words: calcium exchangeable pool, accretion, excretion, gut absorption and bone resorption, collagen synthesis, protein-deprived rats.

Skeletal abnormalities have long been recognized in human protein–calorie malnutrition (PCM). In 1931, Harris observed transverse tabeculation at the growing ends of long bones on X-rays of children suffering from malnutrition. Jones & Dean (1956) described skeletal immaturity and retarded growth of the long bones in children under similar circumstances. A deficiency of cortical bone was claimed by Garn, Rohmann, Behar, Viteri &
Guzman (1964), while Wayburne (1968), in addition demonstrated trabecular bone-loss to be prominent. On the other hand, Reichman & Stein (1968) showed no radiologic abnormalities while Adams & Berridge (1969) suggested that many protein-malnourished children also had varying degrees of rickets which probably complicated the radiological assessment. Higginson (1954), working with autopsy specimens, confirmed thinning and immaturity of the epiphyses of the long bones.

To assess more accurately the dynamics of bone metabolism in PCM and to avoid confusion in the interpretation of such data because of the likely coexistence of vitamin D deficiency, animal models of human PCM have been developed and studied. The protein-depleted pigs of Platt & Stewart (1962) and rats of El-Maraghi, Platt & Stewart (1965) demonstrated similar features to those seen in human PCM, namely rarefaction of long bones, thinner epiphyses and growth retardation.

Kinetic studies have been limited. Shenolikar & Rao (1968) demonstrated reduced calcium accretion rates in protein-deprived rats using $^{45}\text{Ca}$ as a tracer. Jha, Deo & Ramalingaswami (1968) have by means of tetracycline labelling demonstrated a marked reduction of appositional bone growth in the protein-deprived monkey. They noted a reduction of the number of osteoblasts and inconspicuousness of osteoclasts, suggesting a low bone-remodelling rate.

In this present paper, we give the results of a comprehensive study of bone metabolism in the protein-depleted rat. In particular, dynamic aspects of calcium and bone metabolism have been investigated in an attempt to elucidate the possible mechanisms of the skeletal abnormalities discussed above.

**MATERIALS, EXPERIMENTAL DESIGN AND METHODS**

**Rat model**

The studies to be reported were performed on two or more of the following groups of rats.

(i) **Protein-deficient rats.** Five-week-old male Wistar rats were fed a low protein diet (4\% casein) by the 'interval' feeding technique (Leung, Rogers & Harper, 1968) commencing 2 weeks after weaning, for 35 days before study. Young growing rats on 'interval' feeding will adapt to low protein intake without losing their appetite or cutting down on calories. This allows the development of an animal with characteristics very similar to those of human kwashiorkor (Stead & Brock, 1972).

(ii) **Age-matched control rats.** These were fed on a normal protein diet (20\% casein) for 35 days starting 2 weeks after weaning.

(iii) **Weight-matched control rats.** These were studied 2 weeks after weaning when their weights were comparable with the protein-deficient group.

(iv) **Age-matched rachitic rats.** These were studied after being housed in dark cages and fed on a vitamin D-deficient diet rich in calcium and low in phosphorus (Bethke, Kick & Wilder, 1932).

**The diet.** The 4\% and 20\% casein diets contained an equal number of joules/g, the only difference relating to the proportion of protein. Both were adequately supplemented with vitamin D (1\% cod-liver oil), calcium (4-9 g/kg of food) and phosphorus (3-05 g/kg). The animals were housed in wire-bottomed cages, with free access to water, in an air-conditioned room and (with the exception of the rachitic group) received 10 h of ultra-violet light per day from a lamp.
All animals were weighed twice weekly, and their daily food intake was measured carefully. The livers of the protein-deficient rats and age-matched controls were examined histologically after the study of calcium metabolism. Sections were prepared, stained with Haematoxylin and Eosin and examined by light microscopy. Rickets was confirmed in group (iv) by examination of bone histology (see below).

**Serum measurements**

The following serum measurements were made on the protein-deficient rats and age-matched controls:

- Fasting serum calcium by the method of Baron & Bell (1959) based on titration with EDTA, using calceinthymolphthalein as indicator; inorganic phosphorus (Dryer, Tammes & Routh, 1957); alkaline phosphatase by a modification of Bodansky's method (Shinowara, Jones & Reinhart, 1942); serum magnesium, photometrically using Titan Yellow (Neill & Neely, 1956); serum albumin by the biuret method of Fernandez, Sobel & Goldenberg (1966).

**Isotope measurements**

$^{47}$Ca was counted in the intact rat in a small whole-body counter with two opposing photomultiplier tubes connected to a gamma radiation counting system (Nuclear Enterprises) with a counting efficiency of 0.8% and an error of 1%, as described by Le Roith & Pimstone (1973a).

$^{45}$Ca was measured either in 50 µl samples of rat serum (for study of calcium pool, accretion and excretion) or ashed bone (for study of calcium resorption). In all cases the calcium was precipitated as calcium oxalate and incinerated with perchloric acid to remove all inorganic and organic contaminating materials (Humphreys, 1965). To render the calcium perchlorate soluble in toluene, a secondary solvent, tributyl phosphate, was used after which the mixture was added to the primary fluor 2,5-diphenyloxazole in toluene at a concentration of 0.3%. Counting was performed in a Beckman liquid-scintillation counter with an efficiency of 53.4% and an error of less than 5%. Aliquots (2 ml) of the separated $[^{14}$C]hydroxyproline were counted by liquid scintillation after the addition of 0.2 ml of 3 M-HCl and 10 ml of Bray's solution (Firschein, 1969). Correction for quenching was made. Counting efficiency was 67.9% with an error of less than 7%.

**Dynamic studies of calcium metabolism**

**Calcium absorption.** These studies were performed in protein-deficient and age-matched control rats. The techniques used were based on those described by Cameron, Butterfield, Veal, Rees & Parsons (1962), and modified by Tadayyon (1969), in which the gamma emitting radio isotope $^{47}$Ca was used as a tracer. This isotope was introduced by stomach tube into fasting rats and counted immediately in a whole-body gamma counting system as described by Le Roith & Pimstone (1973a). Whole-body counting of the same animals was repeated 3 days later and expressed as a percentage of the original counts. The retention of the isotope thus calculated could be taken to represent calcium absorption. As the total daily excretion of calcium is similar in the protein-deficient and age-matched controls (see later), any difference in percentage retention of the isotope between the groups is likely to be due to altered calcium absorption.

Three different experiments were performed in the two groups of animals: (a) 5 µCi of $^{47}$Ca was administered alone; (b) 5 µCi of $^{47}$Ca was given pre-mixed with a homogenized meal
appropriate to the particular group of rats and introduced intragastrically; (c) 5 μCi of $^{47}$Ca was pre-mixed with homogenized food taken from the diet of the opposite group of rats to assess whether the presence or absence of protein in the meal containing $^{47}$Ca influenced the absorption of the isotope. The numbers of animals used in these experiments are indicated in Table 3.

**Exchangeable calcium pools, calcium accretion and calcium excretion.** These studies were performed on protein-deficient rats (seventeen animals), age-matched controls (fourteen) and weight-matched controls (seven).

The technique used is described by Le Roith & Pimstone (1973a) and is based on the simultaneous intravenous injection of $^{45}$Ca and $^{47}$Ca and the subsequent measurement of fall-off of whole-body $^{47}$Ca retention values and serum $^{45}$Ca specific radioactivity, thus eliminating the need for faecal and urine collection. Calcium excretion thus represents total loss of endogenous calcium by the gut and kidneys.

**Calcium resorption.** This was studied in protein-deficient rats (twenty-four animals) and age-matched controls (twenty-four animals).

The rate of removal of calcium from the long bones was measured by the technique of Firschein & Alcock (1969). The $^{45}$Ca (40 μCi) was injected intravenously into the rats, which were killed at 5-day intervals over a 20-day period. The tibiae were removed, cleaned and ashed in an oven at 600°C for 24 h, then the ash was weighed and dissolved in 1 m-HCl. Aliquots representing 100 mg samples of ashed tibia were counted for $^{45}$Ca radioactivity by liquid-scintillation counting as described above. The mean values of the radioactivity in the tibiae of the groups of rats killed at each time interval were expressed as a percentage of the baseline activity (day 5). The rate of fall-off of radioactive calcium in the ashed tibia represented the rate of removal of the calcium isotope from bone.

**Collagen synthesis.** These studies were performed in protein-deficient rats (seven animals) and age-matched controls (seven animals).

Hydroxyproline, an amino acid found exclusively in connective tissue (Vaughan, 1970) is derived solely from free proline. The rate of collagen synthesis can therefore be estimated by measuring the rate of incorporation of $[^{14}$C]proline into hydroxyproline of long bones as described by Firschein (1969).

$[^{14}$C]Proline (50 μCi) was injected intraperitoneally into the rats which were then killed either 3 or 6 h after injection. The tibiae were removed, cleaned, demineralized with 0.3 m-HCl and then hydrolysed in 6 m-HCl. Hydroxyproline was separated from proline by passage through two Dowex 50W–X8, 200–400 mesh ion-exchange resins. On the first column the hydroxyproline was eluted with 1 m-HCl, and on the second with 0.1 m-citrate buffer, pH 6. Aliquots of hydroxyproline were taken for stable hydroxyproline estimations as well as radioactive assay. The specific radioactivities obtained (expressed as c.p.m. of $^{14}$C/μg of hydroxyproline) gave an indication of the degree of hydroxyproline formed from the labelled proline precursor, and hence of collagen synthesis.

**Direct measurements of bone**

**Extirpated bone weight.** These measurements were performed on protein-deficient rats (twenty-two animals), age-matched controls (twenty-one) and weight-matched controls (eight). Animals were killed and both tibiae and femora removed from each rat. They were then cleaned, and dried in an oven at 125°C for 24 h after which they were carefully weighed.
Extirpated bone ash content. This was measured in eight protein-deficient rats and the same number of age-matched controls. The tibiae and femora previously removed and weighed were divided into three sections. The proximal, mid-shaft and distal fractions were then ashed for 36 h in an oven at 600°C, and the ash weighed. Ash weights were expressed as a percentage of the dry weight of bone.

Radiography. Radiography was performed on protein-deficient rats (sixteen animals) and age-matched controls (sixteen animals). The animals were killed, then the femora and tibiae were removed and cleaned and radiographs were performed using a Phillips Polytome operated at 35 kV, 19 mA with 0.03 s exposures. Cronex film was processed automatically for 90 s by a Kodak Rapid Processor. Total bone-length and epiphyseal width were accurately measured on the radiographs with Vernier calipers.

Microscopic assessment. Sections were made of the decalcified proximal tibial epiphyseal growth plate in the protein-deficient (twelve animals), age-matched control (twelve) and vitamin D-deficient (three groups) rats. The decalcified sections were fixed in formalin, stained with Haematoxylin and Eosin and the epiphyseal width measured. A semiquantitative assessment was made of the extent of unmineralized osteoid, which stained a homogeneous pink.

RESULTS

Rat model

Protein deprivation in the weanling rat is characterized by growth retardation, a low serum albumin, periportal fatty infiltration of the liver, and inactivity of the growth plates at the epiphyseal ends of the long bones (Fig. 2). Details of the complete verification of the identical model appears elsewhere (Stead & Brock, 1972).

Fig. 1 illustrates the growth charts of protein-deprived (4% casein), age-matched (20% casein) and control rats fed ad libitum over the 35-day feeding period. No appreciable weight gain is shown by the protein-deficient group, whereas the age-matched control rats fed on a normal diet by the same technique gain weight at a rate almost equal to the expected growth rate of rats of the same strain fed ad libitum (Fig. 1). The protein-deficient rats do not reduce their food consumption below that of the control group when this is expressed as grams eaten per 100 g body weight (Table 1). Significantly lowered serum albumin concentrations are demonstrated in the protein-deficient rats (Table 2) as well as periportal fatty infiltration of the liver (D. Le Roith & B. L. Pimstone, unpublished work) and narrowed epiphyseal cartilage plates. When comparing the protein-deficient group with the control group, no excessive osteoid accumulation is present in the bones (Fig. 2).

Serum measurements

Table 2 illustrates the significant reduction of total serum calcium, phosphorus and magnesium in the protein-deficient animals compared with age-matched controls. Alkaline phosphatase, however, is unchanged.

Dynamic studies

Calcium absorption. Table 3 shows a significant impairment in calcium absorption in the protein-deficient rat compared with the age-matched control. Both groups of animals show a further proportionate reduction in calcium absorption when the 47Ca is introduced premixed
FIG. 1. The contrast in weight gain between groups of twenty-four rats fed ad libitum (○), 20% casein (■) and 4% casein (●). The mean ± SEM is shown.

TABLE 1. Mean food consumption of twenty-four protein-deprived and twenty-four age-matched control rats, showing no reduction in the proportional food intake of the protein-deprived group

<table>
<thead>
<tr>
<th>Food consumption</th>
<th>Protein-deprived rats (4% casein)</th>
<th>Age-matched controls (20% casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1* 2 3 4 5 6</td>
<td>1 2 3 4 5 6</td>
</tr>
<tr>
<td>g/day</td>
<td>7.9 7.7 7.9 8.1 8.0</td>
<td>6.3 8.0 9.3 10.6 12.2 12.9</td>
</tr>
<tr>
<td>g/100 g body weight</td>
<td>9.6 9.1 9.3 8.8 9.5 9.2</td>
<td>8.8 10.0 9.9 8.6 9.4 8.8</td>
</tr>
</tbody>
</table>

* Weeks on diet.
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with the appropriate meal. Administration of the isotope with a meal from the opposite
group of animals does not alter calcium absorption further.

Exchangeable calcium pools, calcium accretion and calcium excretion. There is a striking and
significant reduction in the size of the calcium pool in the protein-deficient rat as compared

Table 2. Serum chemistry of protein-deprived and age-matched control rats

<table>
<thead>
<tr>
<th>Compound and concentration</th>
<th>Protein-deprived rats (4% casein)</th>
<th>Age-matched control group (20% casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats</td>
<td>Mean values (SEM)</td>
</tr>
<tr>
<td>Albumin (g/100 ml)</td>
<td>20</td>
<td>2.16 (0.05)</td>
</tr>
<tr>
<td>Calcium (mg/100 ml)</td>
<td>13</td>
<td>6.82 (0.40)</td>
</tr>
<tr>
<td>Inorganic phosphorus (mg/100 ml)</td>
<td>14</td>
<td>6.98 (0.20)</td>
</tr>
<tr>
<td>Magnesium (mmol/l)</td>
<td>7</td>
<td>0.07 (0.03)</td>
</tr>
<tr>
<td>Alkaline phosphatase (Shinowara units/litre)</td>
<td>14</td>
<td>32.37 (1.92)</td>
</tr>
</tbody>
</table>

Albumin, calcium, inorganic phosphorus and magnesium are significantly lower in the experimental
group (respectively, $t = 10.704$, $P < 0.001$; $t = 4.674$, $P < 0.001$; $t = 3.065$, $P < 0.01$; $t = 5.201$, $P < 0.001$). Alkaline phosphatase is unchanged ($t = 1.038$, $P < 0.4$).

Table 3. Calcium absorption in protein-deprived and age-matched control rats, as
measured by the percentage whole-body retention of intragastric $^{47}$Ca after 3 days

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Calcium absorption in protein-deprived rats (4% casein)</th>
<th>Calcium absorption in age-matched controls (20% casein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats studied</td>
<td>Mean (SEM)</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>52.04 (3.29)</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>37.40 (2.83)</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>38.32 (2.91)</td>
</tr>
</tbody>
</table>

In experiment 1 both groups received $^{47}$Ca only; in experiment 2 they received
the isotope with a homogenized meal consisting of their appropriate experimental
diet; in experiment 3 the diets with which the $^{47}$Ca was administered were of the
opposite group. In all three experiments a significantly lowered calcium retention
is found in the protein-deprived group (respectively for each experiment, $t = 4.865$, $P < 0.001$; $t = 6.144$, $P < 0.001$; $t = 6.359$, $P < 0.001$).

with both age- and weight-matched control groups (Table 4). The weight-matched controls
that are considerably younger than the other two groups show the largest calcium pool.
The fractional calcium accretion rate is equal in all three groups but because of differences
in calcium pool-size the total daily accretion of calcium is lowest in the protein-deficient rats
and highest in the younger weight-matched controls. A striking finding is a large increase in
the fractional rate of calcium excretion in the protein-deficient rats when compared with both other groups. Consequently, in spite of a substantially smaller pool of calcium, the protein-deficient rats excrete the same amount of calcium a day as the age-matched controls. Because of a very large calcium pool and an equal fractional excretion rate, the young weight-matched control rats excrete a large amount of calcium daily compared with the other two groups.

**Table 4. Exchangeable calcium pool, calcium accretion and excretion in protein-deprived, age- and weight-matched control rats; the mean (and SEM) is shown in each instance**

<table>
<thead>
<tr>
<th></th>
<th>No. of rats</th>
<th>Exchangeable calcium pool (mg)</th>
<th>Calcium accretion</th>
<th>Calcium excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fractional (pool/day)</td>
<td>Absolute (mg/day)</td>
</tr>
<tr>
<td>Protein-derived (4% casein)</td>
<td>17</td>
<td>32.9 (1.96)</td>
<td>65.95 (2.65)</td>
<td>20.70 (0.94)</td>
</tr>
<tr>
<td>Age-matched control group (20% casein)</td>
<td>14</td>
<td>59.81 (3.23)</td>
<td>73.42 (3.84)</td>
<td>45.49 (2.05)</td>
</tr>
<tr>
<td>Weight-matched control group</td>
<td>7</td>
<td>147.93 (15.52)</td>
<td>68.35 (4.30)</td>
<td>98.96 (8.61)</td>
</tr>
</tbody>
</table>

Significant differences between the protein-deprived and age-matched control rats are shown in the exchangeable pool \( t = 7.401, P < 0.001 \), absolute calcium accretion \( t = 11.670, P < 0.001 \) and fractional excretion \( t = 5.018, P < 0.025 \). Significant differences between the protein-deprived and weight-matched control group are shown in exchangeable pool \( t = 11.122, P < 0.001 \), absolute calcium accretion \( t = 12.431, P < 0.001 \) and both fractional and absolute excretion \( t = 8.764, P < 0.001; t = 9.207, P < 0.001 \) respectively.

![Figure 3](image_url)  
**Fig. 3.** Calcium resorption in protein-deprived (○) and age-matched control rats (●) as measured by % retention of \(^{45}\text{Ca}\) in extirpated tibiae at 5-day intervals after intravenous administration of the isotope. Each point represents the mean ± SEM of six observations. Difference between the two groups of rats is significant at 20 days only \( t = 1.928, 0.05 < P < 0.1; t = 1.656, 0.1 < P < 0.2; t = 2.515, P < 0.05; \) at 10, 15 and 20 days).
Bone metabolism in protein deprivation

Fig. 2. Decalcified sections of the growing ends of tibiae of (from left to right) protein-deprived, age-matched control and vitamin D-deficient rats. Note the presence of abundant homogeneous black osteoid and widened cartilage plate in vitamin D deficiency, whereas osteoid is scanty and cartilage plate very narrow in protein deprivation.

(Facing p. 312)
Fig. 6. Comparative radioautographs of tibiae of rats receiving 20% (on left) and 4% casein (on right). The bone from the protein deprived rat is smaller and more radiolucent than the control.
Fig. 4. Incorporation of $[14C]proline$ into tibial hydroxyproline 3 and 6 h after intraperitoneal administration of the isotope in protein-deprived rats ($\circ$; mean, ---) and age-matched controls ($\bullet$; mean, --). A significant decrease in incorporation is seen in the protein-deprived rats after 6 h ($t = 3.612, P < 0.02$).

Fig. 5. A correlation between body and tibial weights in protein-deprived rats ($\circ$), weight-matched ($\bullet$) and age-matched controls ($\bigcirc$). Bone has grown in the protein-deprived rats, in spite of cessation of weight gain; however, bone weight remains below that of the age-matched controls.
Calcium resorption. The rate of calcium resorption from bone is slightly reduced in the protein-deficient rats compared with age-matched controls. However, this reduction is statistically significant only at 20 days (Fig. 3).

Collagen synthesis. Both at 3 and 6 h after injection of $[^{14}C]$proline, its incorporation into hydroxyproline is reduced in protein-deficient rats (Fig. 4). This reduction is statistically significant at 6 h.

Table 5. Bone ash, expressed as % dry weight of bone, in the tibiae and femora of protein-deprived and age-matched control rats; the means (and SEM) are shown

<table>
<thead>
<tr>
<th></th>
<th>Tibia</th>
<th></th>
<th>Femur</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats</td>
<td>Proximal</td>
<td>Midshaft</td>
<td>Distal</td>
</tr>
<tr>
<td>Protein-deprived group (4% casein)</td>
<td>8</td>
<td>48.88</td>
<td>62.73</td>
<td>48.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.96)</td>
<td>(1.0)</td>
<td>(0.86)</td>
</tr>
<tr>
<td>Age-matched controls (20% casein)</td>
<td>8</td>
<td>52.54</td>
<td>63.26</td>
<td>54.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.68)</td>
<td>(0.71)</td>
<td>(0.85)</td>
</tr>
</tbody>
</table>

A significant reduction in bone ash is found only in the proximal and distal portions of the tibiae of the protein-deprived group ($t = 3.941, P<0.005; t = 5.562, P<0.001$ respectively), but the magnitude of change is small.

Table 6. Femoral and tibial lengths in protein-deprived, age- and weight-matched control rats expressed as the mean (and SEM)

<table>
<thead>
<tr>
<th></th>
<th>Bone size</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of rats</td>
<td>Tibia (mm)</td>
<td>Tibial epiphysis (mm)</td>
</tr>
<tr>
<td>Protein-deprived group (4% casein)</td>
<td>16</td>
<td>3.02 (0.03)</td>
<td>0.15 (0.01)</td>
</tr>
<tr>
<td>Age-matched controls (20% casein)</td>
<td>16</td>
<td>3.34 (0.29)</td>
<td>0.21 (0.01)</td>
</tr>
<tr>
<td>Weight-matched controls</td>
<td>8</td>
<td>2.46 (0.04)</td>
<td>0.20 (0.02)</td>
</tr>
</tbody>
</table>

Compared with age-matched controls, the protein-deprived rats show a significant reduction in femoral and tibial length ($t = 6.493, P<0.001; t = 7.397, P<0.001$, respectively), while the epiphysis as % of tibial length is significantly reduced in protein deprivation compared with both control groups ($t = 4.019, P<0.001; t = 5.782, P<0.001$, for age- and weight-matched controls).

Direct measurements on bone

Extirpated bone weight. Fig. 5 compares tibial weight with body weight in the protein-deficient, age-matched and weight-matched control groups. In both control groups of rats bone weight is proportional to body weight. However the protein-deficient rat, although achieving a tibial weight less than expected for age, nevertheless shows a substantial increase in spite of almost total failure to gain body weight.
Extirpated bone ash content. Table 5 contrasts the bone ash of protein-deficient and age-matched controls in the proximal, midshaft and distal tibia and femur. Reduced mineral content (bone ash expressed as a percentage of dry bone weight), is found only at the proximal and distal ends of tibia in the protein-deprived rats. Although these differences are significant, the magnitude of change is not great.

Radiological assessment of total bone and epiphyseal length. Table 6 compares the total length of tibia and femur in the protein-deficient, age- and weight-matched control groups. In addition, the epiphyseal length has been measured separately in the three groups and its proportion of total tibial length separately calculated. Consistent with the data on bone weight, bone length appears greatest in the age-matched control group and least in the younger weight-matched group. The protein-deficient rats show values intermediate between the two groups and significantly different from both. The weight-matched rats show the largest proportional epiphyseal length and this declines with age as shown in the older controls. However, in spite of attaining bone growth intermediate between the two control groups, the protein-deficient animals show the thinnest tibial epiphyses, whether expressed in absolute terms or as a percentage of the total tibial length. A typical example of the contrast between the bones of the protein-deficient and age-matched rats is shown in Fig. 6.

DISCUSSION

Evidence for a metabolic disturbance of bone in human and experimental protein-calorie malnutrition (PCM) has been reviewed in the introduction to this paper. Experimentally, the protein-deprived pig (Platt & Stewart, 1962), monkey (Jha et al., 1968) and rat (Shenolikar & Rao, 1968; El-Maraghi et al., 1965) have been studied by other groups. Some (Platt & Stewart, 1962; El-Maraghi et al., 1965) reported on radiologic and histologic abnormalities while Shenolikar & Rao (1968) described a decrease in calcium accretion. Jha et al. (1968) showed appositional bone growth using tetracycline labelling and commented on the reduction of osteoblastic and osteoclastic activity. We have chosen the rat as an experimental model to study radioactive calcium dynamics more fully, supported by information on hydroxyproline synthesis and direct measurements on extirpated bone.

The validity of the protein-deprived post-weanling rat as an experimental model for human PCM has been demonstrated by Stead & Brock (1972) who have defined the salient chemical, histological and biochemical features. As with their model, our protein-deprived rats show retardation of growth, a lowered serum albumin, narrowed epiphyseal plates and the characteristic fatty infiltration of the liver. Further, no overt rickets is present as the diet contains a normal calcium:phosphorus ratio of 3:2 as well as vitamin D supplementation. However, malnourished children with grossly retarded bone growth rarely show overt rickets, probably due to an inability to synthesize osteoid. It is still conceivable, therefore, that the biological activity of vitamin D may be subnormal in protein deficiency, and partly responsible for some of the effects to be discussed, in the absence of histologic evidence of osteoid accumulation.

The protein-deprived rats have been fed a 4% protein diet with a caloric content equal to the 20% control diet. As their food intake is constant relative to body weight, they have maintained an adequate calorie intake though selectively deprived of protein, and hence have failed to grow.

The serum calcium is low in the protein-deprived rats. To what extent this is due to lowered
calcium binding in the face of a 30% decrease in serum albumin is speculative, as the calcium-binding capacity of serum albumin is not known in the rat. Further, estimations of ionized calcium in serum were not done. These findings contrast, however, with the data of Jha et al. (1968), who, in spite of markedly reduced serum albumin, found normal total serum calcium in protein-deprived monkeys. Hypomagnesaemia is well described in children with PCM and the exact mechanism is not entirely clear but could possibly represent a consequence of poor magnesium intake. On the other hand, as shifts of magnesium and calcium often parallel one another, the changes could be related to the disorder of calcium metabolism discussed below, but this must remain hypothetical. Alkaline phosphatase is normal and would imply fairly adequate osteoblastic activity consistent with our findings of some, although reduced, bone growth. The cause of the lowered inorganic phosphorus is not apparent from our studies but might represent the result of secondary hyperparathyroidism if the lowered serum calcium is due partly to a decrease in the ionized fraction.

Bone growth continues in the protein-deprived rat, albeit at a reduced rate in spite of a total halt in weight gain. This is shown by our data on tibial and femoral length as well as tibial weight. The ability to maintain some bone growth in the face of failure of body weight gain has also been noted in starvation (Sissons, 1956).

Our dynamic studies of bone confirm a low calcium pool and reduced calcium accretion as described by Shenolikar & Rao (1968). This reduction of calcium accretion is associated with a reduced incorporation of proline into hydroxyproline, suggesting an impairment in bone collagen synthesis. However, as relative sizes of the proline and hydroxyproline pools are not known in the control and protein-deprived rats, it is conceivable that the lower incorporation of proline in protein deficiency could be a reflection of dilution of the isotope in a larger pool. In spite of a reduction in calcium pool and calcium accretion, the fractional excretion rate of calcium is elevated so that the total amount of calcium excreted per day in the urine and faeces is not reduced below normal. This might in small part be due to increased loss of endogenous calcium secreted into the bowel and poorly absorbed as shown by Shenolikar & Rao (1968). On the other hand, this may reflect an impaired renal tubular reabsorption of calcium so that the loss of calcium via the renal route forms a greater proportion than normal of total calcium excretion. However, as our techniques do not allow separate assessment of the urine and faecal calcium loss, it is not possible to clarify this point further.

Direct measurement of calcium resorption is only very slightly lowered in the protein-deprived rat suggesting relative excess resorption in the face of lowered calcium accretion. The failure to reduce calcium resorption may be interpreted as a failure of reduction of total bone resorption since both mineral and matrix are always resorbed simultaneously (Urist, 1969). This, together with the more strikingly reduced accretion of calcium and synthesis of collagen, results in the impaired bone growth and reduced cortical bone which has been demonstrated in human PCM (Garn et al., 1964) and has been shown in our rats by bone densitometry (Le Roith & Pimstone, 1973b). This is not accompanied by change in quality of bone, as suggested by the normal percentage ash content of the extirpated bone and confirmed by densitometric data (Le Roith & Pimstone, 1973b). Similar findings have been reported in pigs by Platt & Stewart (1962).

Finally, we have demonstrated that a significant reduction in intestinal calcium absorption is present in protein deprivation whether the isotope is given alone, or with a meal. The latter further lowers the absorption in both groups of animals, presumably by providing a stable
calcium load and ‘diluting’ the isotope. The consistently lower absorption in the protein-deficient animals is not merely related to the amount of protein in the food in which calcium is being administered, as a single meal containing adequate protein did not improve the absorption of radioactive calcium in the chronically protein-deprived group. McCance, Widdowson & Lehmann (1942) reported greatly improved calcium balance in five volunteers when changed from periods of low to high protein intake. They concluded that little calcium would be absorbed if the diet contained no protein or amino acids. It has recently been shown that active calcium absorption in the gut requires the synthesis of a specific calcium binding protein (Wassermann & Taylor, 1966) probably under the control of 1,25-dihydroxycalciferol (Norman, Myrtle, Midgett, Nowick & Popjak, 1971). In the protein-deficient animal it is quite conceivable that the synthesis of this important carrier protein might be impaired, possibly due to an altered biologic activity of vitamin D, and the active transport of calcium thus prejudiced.

The precise sequence of events culminating in the metabolic disturbance of bone in the protein-deficient rat can only be speculative. It is possible that the fundamental abnormality might be a defect of calcium transport which, at least in the gut, depends on the synthesis of a specific binding protein. Impaired gut absorption of calcium would give rise to a smaller calcium pool and lowered accretion and hence bone formation. Lowered collagen synthesis, possibly as an independent feature of protein deficiency, would compound this. Bone resorption, nevertheless, is reduced only slightly in the face of these disturbances. Even though resorption is low relative to formation in the young rat, failure to cut back resorption, possibly as a result of an attempt to maintain the extra osseous calcium pool and serum calcium concentration, may contribute to the reduction of bone in protein deficiency. The fractional excretion of calcium is high in the protein-deprived rat. Our technique, as already indicated, cannot distinguish between faecal and urinary excretion. Endogenous faecal calcium has been shown to be elevated by Shenolikar & Rao (1968). However, elevation of urinary calcium excretion might conceivably be significant. The recent demonstration by Taylor & Wassermann (1970) of calcium-binding protein in the kidney may imply that disturbances in renal tubular calcium transport may also be present in protein deficiency.

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