CITRATE IN MINERALIZED TISSUES—VI

THE EFFECTS OF DIETARY REHABILITATION WITH CALCIUM OR WITH VITAMIN D, OR WITH CALCIUM AND VITAMIN D, IN RATS PREVIOUSLY MAINTAINED ON A DIET DEFICIENT IN BOTH CALCIUM AND VITAMIN D

R. L. HARTLES, A. G. LEAVER and J. T. TRIFFITT

Biochemistry Department, School of Dental Surgery, University of Liverpool

Summary—Fifty weanling hooded rats were maintained for 8 weeks on a diet low in calcium and vitamin D. Similar groups were then transferred to diets adequate in calcium, or adequate in vitamin D, or sufficient in both respects. They were continued on these diets and representatives were killed at intervals during the 35 day period. The concentrations of calcium, phosphorus, and citrate in blood and bone were determined.

When the deficient animals were rehabilitated with vitamin D alone, the concentration of citrate in bone was increased before that in the serum. Rehabilitation with calcium alone caused an increase in the concentration of serum citrate before any increase was observed in the bone.

When the animals were transferred to a diet containing adequate supplies of calcium and vitamin D, the effects of both factors were distinguished since they occurred at different times.

The implications of these results in relation to the possible function of citrate in bone are discussed.

INTRODUCTION

SEVERAL WORKERS have shown that the consumption of diets low in calcium and deficient in vitamin D results in a lessened accumulation of citrate in the bone of the rat. The bone citrate can be increased by adding either vitamin D or calcium to the diet consumed by the rat (STEENBOCK and BELLIN, 1953; CARLSSON and HOLLUnger, 1954; HARTLES and LEAVER, 1961a,b). Thus although the citrate content of bone may be increased by the presence of vitamin D, it is not appreciably depressed in the absence of the vitamin provided that the calcium nutrition of the animal is adequate. The present study was designed to investigate the effects of selective and complete rehabilitation of the diet of young rats previously maintained for 8 weeks on a diet deficient in both calcium and vitamin D. Samples of bone and blood were obtained by killing animals at intervals during the rehabilitation period of 35 days. The samples were analysed for calcium, inorganic phosphorus, and citrate.
EXPERIMENTAL

Animals and diets

Four groups, each of fifty 1-month-old black and white hooded rats from our own colony, were placed on a diet containing 0.026 per cent calcium, 0.45 per cent phosphorus and no added vitamin D. The diet (HS10B) was similar to diet HS10 already described in detail (Hartles and Leaver, 1961a) except that it contained blood albumin instead of egg albumin. The four groups of animals were maintained on the doubly-deficient diet for a period of eight weeks. Ten animals from each group were then anaesthetised with ether and killed by decapitation. The remaining animals were treated as follows:

Group 1. Forty animals, remained on diet HS10B.

Group 2. Forty animals, transferred to diet HS9B, which contained 0.56 per cent calcium, 0.42 per cent phosphorus but no ergocalciferol.

Group 3. Forty animals, transferred to diet HS8B, which contained 0.026 per cent calcium, 0.45 per cent phosphorus and 200 μg ergocalciferol in each 960 g of diet.

Group 4. Forty animals, transferred to diet HS7B, which contained 0.56 per cent calcium, 0.42 per cent phosphorus and 200 μg ergocalciferol in each 960 g of diet.

The main features of the rehabilitation diets were as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Calcium</th>
<th>Phosphorus</th>
<th>Vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Deficient</td>
<td>Normal</td>
<td>Deficient</td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>Normal</td>
<td>Deficient</td>
</tr>
<tr>
<td>3</td>
<td>Deficient</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Duration of rehabilitation

Ten animals from each group were killed at 2, 7, 16, and 35 days after transfer to the rehabilitation diets. The animals were weighed weekly and immediately before being killed.

The use of blood albumin as dietary protein

In earlier experiments egg albumin was used as the source of dietary protein (Hartles and Leaver, 1961a,b, 1962). This proved perfectly satisfactory for experimental periods of 10–12 weeks from weaning. After longer periods control animals showed signs of abnormality; bald patches developed, and ulceration of lips and anal region were observed. It was considered that these signs might be caused by a biotin deficiency precipitated by the consumption of the uncooked egg albumin. When rats were maintained on diets containing blood albumin no such signs were observed. In view of these findings, the diets in the present study were prepared with blood albumin. It was also found that whereas the calcium and phosphorus content of the bone was unaffected by the change in protein, the bone citrate was raised irrespective of the state of calcium or vitamin D nutrition but the relative differences were maintained. A preliminary report of these findings has already been made (Leaver, Hartles and Triffitt, 1962).
Blood analysis

The rat was anaesthetized by ether inhalation, decapitated and the blood collected in a glass funnel suspended over a 15 ml centrifuge tube; 0·1 ml of a solution of heparin (10³ i.u./ml) was added to the base of the interior of the funnel. In this fashion up to 8 ml of blood was collected from the largest rats. The tube was quickly shaken to mix the blood with the heparin and centrifuged at 3000 rev/min for 10 min. The serum was removed carefully, with a graduated pipette and transferred to a clean dry centrifuge tube. One quarter of the serum volume of trichloracetic acid (20 % w/v) was added to precipitate protein, the mixture allowed to stand for 10 min and then centrifuged at 3000 rev/min for 10 min. The supernatant was used for the analysis of calcium, inorganic phosphate, and citrate.

**Calcium.** Determinations were carried out on 0·2 ml samples of supernatant by the murexide complexometric method, described under bone analysis.

**Phosphorus.** Inorganic phosphate was determined on 0·1 ml samples of supernatant by the method previously described for bone (HARTLES, 1951).

**Citrate.** The analysis was a modification of the method described by McARDLE (1955). The sample, containing 10–100 μg citric acid (0·5–1·0 ml of supernatant), was placed in a 10 ml graduated ground-glass stoppered tube and the volume made up to 1 ml. Sulphuric acid (1·5 ml, 27 N) was added, the mixture shaken and cooled to room temperature. The bromide-bromate-vanadate solution (1 ml) was added, the tube stoppered, shaken, and allowed to stand for 45 min; 1 ml of ferrous sulphate solution was added, the contents mixed thoroughly and the stoppers removed. The tube was allowed to stand for 5 min and the bromine vapours above the liquid were removed by suction with a water pump. Heptane (2·5 ml) was now added and the tube shaken for 90 sec and allowed to stand until the two layers had separated. A sample (2 ml) of the heptane layer was removed with a pipette, transferred to a 15 ml centrifuge tube containing a little anhydrous sodium sulphate and allowed to stand for 2 min. The heptane was decanted into a 10 ml stoppered test tube containing 1·8 ml of a solution containing thiourea and borax and 0·2 ml of sodium sulphide solution. The tube was shaken carefully for 1 min before standing in the dark for 15 min. The solution was now transferred into a micro-cuvette and the intensity of the yellow colour determined with a “Spekker” absorptiometer using distilled water as a blank with an Ilford 601 violet filter. Each individual blood sample was analysed in triplicate. Standards containing 20, 40, 60 μg of citric acid were determined at the same time.

**Reagents.** Standard citric acid solution: 100 μg/ml.

Sulphuric acid, 27 N; 75 ml. H₂SO₄ (36 N) added to 25 ml distilled water.

Bromide-bromate-vanadate reagent: 19.84 g KBr, 4.92 g KBrO₃, 12 g NH₄VO₃ dissolved and made up to 1 l. with distilled water.

Ferrous sulphate: 200 g FeSO₄.7H₂O dissolved and made up to 1 l. with N.H₂SO₄.

n-Heptane: British Drug Houses Ltd.
Thiourea/borax reagent: 2 g borax (sodium tetraborate) dissolved in 100 ml distilled water, 9.0 g thiourea dissolved in this without heating. Stored in the refrigerator.

Sodium sulphide: 2.0 g Na₂S.9H₂O dissolved in 100 ml distilled water. Stored in the refrigerator.

Bone analyses

The femora were dissected as cleanly as possible, pairs of bones from each animal were wrapped in filter paper and extracted continuously with boiling ethanol in a Soxhlet extractor for 18 hr. The fat-free bones were scraped gently to remove the last traces of soft tissue, dried at 100°C for 3 hr, and weighed. The dry fat-free bones were fragmented in a percussion mortar and the fragments shaken for 4 min in an agate micro-ball mill. This treatment produced a powdered bone which passed through a 60-mesh sieve, it was separated into two fractions by flotation in a bromoform-acetone mixture of specific gravity (sp.gr.) of 1.5. The fraction of sp.gr. ≥1.5 was used in all bone analyses. The samples were weighed, treated with N.HCl, filtered after standing for 5 min and made up to a volume of 10 ml with NHCl. Samples of this solution were used for the determination of citric acid. The solution was diluted 100 times with distilled water for the determination of calcium and phosphorus.

Calcium. The determination of calcium was carried out by the murexide complexometric method using titration with ethylenediamine tetraacetate (EDTA) in an “Eel” titrator with orange “Eel” filter No. 606. A sample (0.3-1.0 ml) of the diluted solution of bone mineral containing approximately 0.05 mg. Ca was added to 2 ml of murexide solution (6 mg/100 ml), to this was added 0.2 ml N NaOH, and the solution titrated to maximum galvanometer reading with the EDTA solution (50 mg/100 ml). A suitable standard containing 0.05 mg Ca/ml was prepared by dissolving 1.25 g calcium carbonate (analytical grade) in NHCl, making up to 1 l. and diluting ten times for use.

Phosphorus. Inorganic phosphate was determined as previously described (Hartles, 1951).

Citrate. The determination of bone citrate was carried out as previously described (Hartles and Leaver, 1961a) except that the heptane extract was shaken with sodium sulphide solution in 15 ml ground-glass stoppered tubes instead of in separating funnels. After standing for 10 min the aqueous layer was withdrawn with a pipette.

Ash values. Individual humeri were rendered dry and fat-free in the same way as the femora. The weighed humeri were ashed by heating in crucibles in a muffle furnace for 8 hr at 500°C, cooled, weighed, and reheated to constant weight.

RESULTS

The rats maintained on diets HS10B (low Ca, low vitamin D) grew moderately well during the 8-week period following weaning, but during the 5-week continuation period (group 1) the rate of growth was much less rapid.
The animals of group 2 on transference to diet HS9B (adequate Ca, low vitamin D) grew well and at an increased rate during the 5-week period.

The growth of group 3 animals when transferred to diet HS8B (low Ca, adequate vitamin D) was no better than that of the rats of group 1.

In group 4, the animals when transferred to diet HS7B (adequate Ca, adequate vitamin D) grew well but no better than those in group 2. Thus in the presence of adequate dietary calcium the addition of vitamin D did not improve growth, nor did the addition of the vitamin aid growth when the intake of calcium was low. Final body weights are given in Tables 1-4.

**General condition of animals**

The animals in groups 2, 3 and 4 remained active, their fur was in good condition and there were no signs of difficulty in locomotion. Two animals in group 1 showed some signs of paralysis of the hind quarters on the penultimate day of the continuation period of 5 weeks.

**Relation between femoral and general growth**

The ratio of femoral weight to body weight is shown in Tables 1-4 and is designated F/T. For the animals of group 2, rehabilitated with calcium, the ratio increased steadily for the male rats, showing that growth of the femur occurred at a rate greater than that for the whole body. In the females there was a fall in the ratio in animals rehabilitated for 2 days, but thereafter it increased steadily at a rate slightly but consistently greater than for the males. It appears, therefore, that the response to rehabilitation with calcium was, in the males, the refurbishment and growth of the femora at a rate in excess of the increase of body weight. For the females, the first response was to increase body weight more quickly than femoral weight but after 7 days this situation was reversed and the ratio of femoral weight to body weight increased.

In group 4 animals (Table 4) which were rehabilitated with calcium and vitamin D there were slight differences compared with group 2 animals. In the males the ratio F/T remained constant after 2 days on the diet HS7B, thereafter rising in much the same way as in the animals of group 2. In the females there was again a fall in the ratio after 2 days but subsequently it exceeded that in the male animals.

When the corresponding data are compared for groups 1 and 3 (Tables 1 and 3) it can be seen that there was only a slight tendency in the animals of group 1 (low Ca, low vitamin D) for femoral growth to be relatively greater than the increase in body weight. In group 3 animals, rehabilitated with vitamin D alone, there was no such tendency.

**Bone fraction of sp.gr. ≥1.5**

In groups 1 and 3 (Tables 1 and 3) the low-calcium diets resulted in the production of femora of small mass, only one quarter of each bone had a sp.gr. ≥1.5, as shown by the ratio B/F. The presence or absence of vitamin D seems to have little effect after 5 weeks continuation, except that in the presence of the vitamin the gross weights of the dry fat-free femora were slightly less than in its absence. This difference was, however, not statistically significant (P> 0.05).
In groups 2 and 4 (Tables 2 and 4) the situation was different. The weights of the femora in the two groups were similar but the proportion of material obtainable of sp.gr. ≥1.5 was significantly greater (P < 0.01) for the males when the animals were rehabilitated with both calcium and vitamin D than with calcium alone. The difference between females was not significant.

Analytical data—Serum

The results are presented in Tables 1–4.

Calcium. After 8 weeks on the diet low in calcium and vitamin D (HS10B), the rats had serum calcium values in the region of 75–85 μg/ml.

Rehabilitation with calcium alone (group 2) resulted in a rapid and sustained rise in serum calcium.

Transference to a diet containing vitamin D but low in calcium (group 3) resulted in a comparatively small but statistically significant rise (P < 0.01) in serum calcium, which reached its maximum value after 16 days.

Rehabilitation with both calcium and vitamin D (group 4) resulted in a rapid rise in serum calcium similar to that in group 2 animals.

Phosphorus. The pattern of response of the serum phosphate to rehabilitation was not easily perceived. The animals in groups 2 and 4, killed after 8 weeks depletion period had lower values for serum phosphate than the corresponding animals in groups 1 and 3, although they all received the same diet HS10B. It seems, therefore, that in these circumstances, control of serum phosphate is more disturbed than is serum calcium. At the end of the 5-week continuation period the serum phosphate values for the animals of group 1 (low Ca, low vitamin D) were slightly higher, and the values for group 3 (low Ca, adequate vitamin D) were slightly lower than at the beginning of the period.

Rehabilitation with calcium and vitamin D (group 4) resulted in a rise in serum phosphate at the end of the 5 week continuation period (Table 4). In group 2 animals which only received additions of calcium, the serum phosphate rose by the sixteenth day but fell again in the animals killed after 35 days.

Citrate. Animals maintained for 8 weeks from weaning on diet HS10B (low Ca, low vitamin D) had serum citrate values between 24–34 μg/ml which rose slowly over the 5-week continuation period to values of 42 μg/ml (Table 1).

Rehabilitation with vitamin D alone (group 3) resulted in a steady rise in serum citrate which was greatest in those animals killed after 16 days and declined after 35 days (Table 3).

Rehabilitation with calcium alone (group 2) was accompanied by a rise in serum citrate which was greatest in those animals killed after 2 days. The citrate returned to the initial value after 7 days (Table 2).

When the animals were given diets containing adequate calcium and vitamin D (group 4), maxima in serum citrate were observed at 2 and 16 days (Table 4). Each of these maxima corresponded in time with those individually observed in groups 2 and 3 respectively.
Analytical data—Bone

All data for ash values refer to the portion of bone of sp.gr. ≥ 1.5.

Calcium. Diets deficient in calcium and vitamin D produced femora of small mass and with only a low proportion of bone of sp.gr. ≥ 1.5. After 8 weeks, this fraction of the bone contained about 20–21 per cent calcium. During the 5-week continuation period (group 1) the calcium content fluctuated but finally was slightly lowered (Table 1).

Transference to a diet containing adequate calcium (group 2) resulted in a sustained increase in calcium content, at the end of the 35 day period the bone fraction contained over 24 per cent Ca (Table 2).

Rehabilitation with vitamin D alone (group 3) again resulted in fluctuations in calcium content of the bone fraction during the 35-day period, but the final analysis showed no change from the initial value (Table 3).

The bones of group 4 animals showed a steady increase in calcium content when the rats were transferred to a diet adequate in calcium and vitamin D. After 35 days the bone fraction contained 25 per cent Ca.

### Table 1. Average values for data obtained from animals of group 1 maintained on diet HS10B for 8 weeks after weaning and then continued for periods of up to 35 days on the same diet (low Ca, no added vitamin D)

<table>
<thead>
<tr>
<th>No. of days on continuation diet</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>16</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal body weight (T)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g)</td>
<td>125</td>
<td>116</td>
<td>129</td>
<td>144</td>
<td>161</td>
</tr>
<tr>
<td>(g)</td>
<td>125</td>
<td>98</td>
<td>116</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>Weight of paired femora (F)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg)</td>
<td>338</td>
<td>325</td>
<td>347</td>
<td>376</td>
<td>453</td>
</tr>
<tr>
<td>(mg)</td>
<td>322</td>
<td>295</td>
<td>339</td>
<td>384</td>
<td>414</td>
</tr>
<tr>
<td>Bone fraction of sp.gr. ≥ 1.5 (B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mg)</td>
<td>54.1</td>
<td>28.9</td>
<td>60.9</td>
<td>61.28</td>
<td>129</td>
</tr>
<tr>
<td>(mg)</td>
<td>72.7</td>
<td>32.9</td>
<td>57.6</td>
<td>71.0</td>
<td>99.1</td>
</tr>
<tr>
<td>(B/F) × 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g)</td>
<td>15.0</td>
<td>8.6</td>
<td>16.0</td>
<td>15.7</td>
<td>27.5</td>
</tr>
<tr>
<td>(g)</td>
<td>15.5</td>
<td>10.3</td>
<td>16.4</td>
<td>18.5</td>
<td>23.3</td>
</tr>
<tr>
<td>(F/T)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g)</td>
<td>2.73</td>
<td>2.82</td>
<td>2.75</td>
<td>2.71</td>
<td>2.82</td>
</tr>
<tr>
<td>(g)</td>
<td>2.57</td>
<td>3.05</td>
<td>2.94</td>
<td>2.75</td>
<td>2.96</td>
</tr>
<tr>
<td>Serum Ca (µg/ml)</td>
<td>77.3</td>
<td>72.0</td>
<td>70.8</td>
<td>79.3</td>
<td>80.5</td>
</tr>
<tr>
<td>Serum P (µg/ml)</td>
<td>89.1</td>
<td>82.2</td>
<td>70.5</td>
<td>90.8</td>
<td>94.1</td>
</tr>
<tr>
<td>Serum citrate (µg/ml)</td>
<td>34.1</td>
<td>35.6</td>
<td>43.0</td>
<td>42.7</td>
<td>41.9</td>
</tr>
<tr>
<td>*Bone Ca (mg/100 mg)</td>
<td>21.7</td>
<td>21.3</td>
<td>23.3</td>
<td>22.3</td>
<td>20.9</td>
</tr>
<tr>
<td>*Bone P (mg/100 mg)</td>
<td>8.97</td>
<td>8.64</td>
<td>9.57</td>
<td>9.60</td>
<td>9.47</td>
</tr>
<tr>
<td>*Bone citrate (mg/100 mg)</td>
<td>0.70</td>
<td>0.53</td>
<td>0.77</td>
<td>0.69</td>
<td>0.65</td>
</tr>
<tr>
<td>*Bone Ca/P</td>
<td>2.42</td>
<td>2.47</td>
<td>2.43</td>
<td>2.32</td>
<td>2.21</td>
</tr>
<tr>
<td>Bone ash (%) (whole humerus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>41.8</td>
</tr>
</tbody>
</table>

* Bone fraction of sp.gr. ≥ 1.5. † S.E.M.
TABLE 2. AVERAGE VALUES FOR DATA OBTAINED FROM ANIMALS OF GROUP 2 MAINTAINED ON DIET HS10B FOR 8 WEEKS AFTER WEANING AND THEN CONTINUED FOR PERIODS OF UP TO 35 DAYS ON DIET HS9B (NORMAL Ca, NO ADDED VITAMIN D)

<table>
<thead>
<tr>
<th>No. of days on continuation diet</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>16</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal body weight (T) (g)</td>
<td>♂ 114</td>
<td>135</td>
<td>153</td>
<td>193</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>♀ 106</td>
<td>119</td>
<td>124</td>
<td>151</td>
<td>165</td>
</tr>
<tr>
<td>Weight of paired femora (F) (mg)</td>
<td>♂ 276</td>
<td>350</td>
<td>419</td>
<td>585</td>
<td>691</td>
</tr>
<tr>
<td></td>
<td>♀ 269</td>
<td>285</td>
<td>366</td>
<td>494</td>
<td>596</td>
</tr>
<tr>
<td>Bone fraction of sp.gr. 1·5 (B) (mg)</td>
<td>♂ 29·9</td>
<td>87·0</td>
<td>104</td>
<td>149</td>
<td>228</td>
</tr>
<tr>
<td></td>
<td>♀ 37·6</td>
<td>68·1</td>
<td>95·7</td>
<td>148</td>
<td>252</td>
</tr>
<tr>
<td>(B/F) × 100</td>
<td>♂ 10·8</td>
<td>24·8</td>
<td>24·1</td>
<td>25·6</td>
<td>32·3</td>
</tr>
<tr>
<td></td>
<td>♀ 14·5</td>
<td>23·4</td>
<td>26·3</td>
<td>29·4</td>
<td>42·5</td>
</tr>
<tr>
<td>(F/T)</td>
<td>♂ 2·38</td>
<td>2·56</td>
<td>2·78</td>
<td>3·05</td>
<td>3·22</td>
</tr>
<tr>
<td></td>
<td>♀ 2·49</td>
<td>2·39</td>
<td>2·93</td>
<td>3·36</td>
<td>3·63</td>
</tr>
<tr>
<td>Serum Ca (µg/ml)</td>
<td>87·1 ± 1·3†</td>
<td>124 ± 1·8</td>
<td>136 ± 2·6</td>
<td>134 ± 1·7</td>
<td>127 ± 1·4</td>
</tr>
<tr>
<td>Serum P (µg/ml)</td>
<td>64·6 ± 3·7</td>
<td>69·5 ± 3·8</td>
<td>83·3 ± 3·9</td>
<td>86·9 ± 3·4</td>
<td>73·3 ± 4·5</td>
</tr>
<tr>
<td>Serum citrate (µg/ml)</td>
<td>23·7 ± 1·6</td>
<td>59·0 ± 2·7</td>
<td>25·4 ± 2·3</td>
<td>25·8 ± 2·2</td>
<td>19·9 ± 1·98</td>
</tr>
<tr>
<td>Bone Ca (mg/100 mg)</td>
<td>19·5 ± 0·33</td>
<td>21·9 ± 0·20</td>
<td>22·6 ± 0·13</td>
<td>24·0 ± 0·17</td>
<td>24·4 ± 0·10</td>
</tr>
<tr>
<td>Bone P (mg/109 mg)</td>
<td>81·7 ± 0·25</td>
<td>87·4 ± 0·14</td>
<td>10·1 ± 0·29</td>
<td>10·8 ± 0·11</td>
<td>11·6 ± 0·13</td>
</tr>
<tr>
<td>Bone citrate (mg/109 mg)</td>
<td>0·63 ± 0·09</td>
<td>1·02 ± 0·05</td>
<td>1·22 ± 0·06</td>
<td>1·13 ± 0·02</td>
<td>0·96 ± 0·01</td>
</tr>
<tr>
<td>Bone Ca/P</td>
<td>2·38</td>
<td>2·41</td>
<td>2·23</td>
<td>2·21</td>
<td>2·11</td>
</tr>
<tr>
<td>Bone ash (%) (whole humerus)</td>
<td>57·5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Bone fraction sp.gr. 1·5. † S.E.M.

Phosphorus. Continued maintenance on diets low in calcium and vitamin D (group 1) appeared to result in a small increase in the phosphorus content of the bone fraction (Table 1).

The animals of group 3, rehabilitated with vitamin D only, showed little change in bone phosphate (Table 3).

On transference to the diet adequate in calcium (group 2) the bone fraction showed a steadily increasing phosphorus content (Table 2). The corresponding values for group 4 animals which received adequate calcium and vitamin D (Table 4) also showed a steady increase in phosphate concentration.

Citrate. Rats maintained on diets deficient in both calcium and vitamin D for 8 weeks had bone fractions containing 0·6-0·7 per cent citrate, this value fluctuated during the 35-day continuation period but finally there was little change (Table 1).

Transference to the diet containing adequate calcium (group 2) resulted in a rise in the concentration of bone citrate with a maximum value observed after 7 days, the values then declined but remained above the initial figure (Table 2).
When the rats were rehabilitated with vitamin D alone (group 3) the bone citrate concentration rose significantly in 2 days and then declined to the initial value after 17 days (Table 3).

Diets adequate in calcium and vitamin D (group 4) produced a rise in bone citrate concentration after 2 days, the values then declined slowly to a level above the initial value (Table 4).

**Bone ash.** Analyses for ash were carried out on the humeri of rats which had survived the whole experimental period. The mean values for the bones of animals on the low calcium diets were about 40 per cent. For the animals which received adequate calcium the values were about 58 per cent. The presence or absence of added vitamin D did not appear to affect bone-ash values in the circumstances of our experiments.

**DISCUSSION**

Analyses were carried out on the fraction of the bone of specific gravity greater than 1.5. This is an arbitrary line of demarcation but we hope that our separation technique will have removed unmineralized tissue and that our analytical figures will provide data that will be representative of the actual bone that has been formed.
In the circumstances of our experiments, when the intake of calcium was low the presence or absence of vitamin D had little effect on growth. Similarly when the diets contained adequate amounts of calcium the presence of vitamin D did not increase either the rate of growth or the terminal body weight of the animals. This result is contrary to the observation of Harrison, Harrison and Park (1958) who found a diminished rate of growth in their animals which received diets lacking in vitamin D. It is difficult to reconcile these two conflicting observations; it is unlikely that our diet HS10B contained appreciable amounts of the vitamin because of the response observed in the blood and bone chemistry of rats transferred to diet HS8B which provided only a physiological amount of the vitamin.

In the rats receiving diets deficient in calcium, those to whom vitamin D was restored for 35 days appeared to have slightly less heavy femora (Table 3) than those deprived of the vitamin (Table 1). The difference, however, was not significant. The proportion of the femora of sp.gr. ≥1.5 recovered was similar in each group.

It was noticeable that the rats which were transferred to diets adequate in calcium increased their femoral weights at a rate greater than the increase in body weight irrespective of the presence or absence of vitamin D. However, after 35 days
the proportion of the femora of sp.gr. ≥1·5 recovered was, in the males, significantly
greater in those animals receiving the vitamin (P < 0·01) than those in which depriva-
tion was continued. The difference observed in the female rats was not significant.
Thus it would appear that after a period of depletion of calcium and vitamin D,
there is some indication that remineralization of the long bones may be more
adequately achieved when both calcium and vitamin D are restored to the diet
than when calcium alone is added. However, this conclusion is not supported by
our observation that ash values in both groups were similar. It is known that a
simple deficiency of vitamin D does not cause rickets in the young rat; nevertheless,
HARRISON and FRASER (1960) report that there are certain histological changes in
rat bone when the animals are deprived of the vitamin even though the diet may
contain a sufficiency of calcium. Our results show that in the more highly mineralized
fraction of the femur there was a slightly but significantly higher proportion of
calcium (P < 0·05) and phosphorus (P < 0·01) in those animals which had been
rehabilitated for 5 weeks with a diet containing adequate calcium and vitamin D
than in those which had received calcium alone.
In the animals which received diets low in calcium, the presence of vitamin D
during the 5-week continuation period did not cause any significant change in the
calcium and phosphorus content of the portion of bone analysed.

Citrate in blood and bone

The main object of this study was to investigate the effects on blood and bone
citrate of adding calcium or vitamin D, or both calcium and vitamin D to the diets
of rats previously maintained for 8 weeks after weaning on diets deficient in both
calcium and vitamin D. All diets contained adequate amounts of phosphorus.

Citrate is present in relatively large amounts in the skeletons of many species
(THUNBERG, 1953). DICKENS (1941) first demonstrated the presence of citrate in bone,
and calculated that about 70–90 per cent of the citrate present in the body at any one
time was contained in the skeleton. It is of the greatest interest therefore to enquire
whether or not citrate has any function to perform in the maintenance of bone or
in calcium metabolism. It seems established that both the state of calcium nutrition
and of vitamin D intake influence the accumulation of citrate in bone (STEENBOCK
and BELLIN, 1953; CARLSSON and HOLLUNGER, 1954; HARTLES and LEAVER, 1961a,b).
It is less clear how this influence is exerted.

In the animals deprived of adequate calcium and vitamin D (group 1) there
appeared to be a slight but significant increase in serum citrate concentration from
the eighth to the thirteenth week (P < 0·01). There was no such overall increase
in bone citrate concentration although there was considerable variation in the values
obtained from animals killed at the intermediate times (Table 1). In those animals
which received adequate dietary calcium (Tables 2 and 4) the final values for serum
citrate concentration were much lower irrespective of the presence or absence of
vitamin D. It is possible therefore that the slow increase in serum citrate concen-
tration in group 1 is a response to the increasingly severe lack of calcium and not to
a deficiency of vitamin D.
In the animals of group 3 which were transferred to a diet containing adequate vitamin D there was a rise in serum citrate concentration which was greatest in those animals killed after 16 days. The final figure for serum citrate was not significantly different from that in the animals lacking both calcium and the vitamin (Tables 1 and 3). The rise in serum citrate in response to vitamin D appeared to be superimposed upon the slow rise observed in group 1 animals.

The greatest rise in bone citrate concentration in group 3 animals in response to vitamin D administration occurred after two days rehabilitation, thereafter it returned to the initial value (Table 3). In these circumstances there seems to be some association between serum citrate and serum calcium concentration which is not apparent in any other group. The fact that the maximum bone citrate concentration occurred before maximum blood citrate suggests that citrate was being formed locally in the bone in response to the administration of vitamin D and was not a result of humeral conditions. The observations further suggest that in conditions of severe calcium deficiency the presence of vitamin D results in a temporary increase in the synthesis of bone citrate which aids to a limited extent the mobilization of calcium from the skeleton to the blood.

Turning now to the effect of rehabilitating animals with the diet containing adequate calcium but still deficient in vitamin D (Table 2), several interesting facts emerged. In 2 days there was a great increase in serum calcium and a large peak increase in serum citrate concentration. The serum calcium continued to rise until the seventh day and was then maintained at an approximately normal value. The serum citrate declined rapidly to a low value. It is possible that this large and rapid rise in serum citrate concentration is associated with the rapid transference of calcium across the intestinal wall, and that when the serum calcium has been restored to normal the production of citrate falls away.

The bone citrate concentration increased after 2 days, but the greatest value was observed after 7 days; thereafter it declined slightly (Table 2). Thus in circumstances where remineralization of the skeleton is taking place, citrate concentrations can be raised in both serum and bone, even when there is a deficiency of vitamin D.

In the group 4 animals rehabilitated with both calcium and vitamin D there was again a rapid rise in both serum calcium and citrate after two days (Table 4). The pattern of increase in serum calcium was broadly similar to that in group 2 animals (low vitamin D, normal Ca, Table 2) except that there was a slight fall in value after 16 days. There was, however, a major difference in serum citrate, namely a second peak value after 16 days, almost as high as that observed after two days rehabilitation. The maximum concentration of bone citrate was observed after 2 days.

It is possible that we are observing here two distinct effects; the first being the response to rehabilitation with calcium, which results in a rapid increase in serum calcium and citrate followed by remineralization of the skeleton with a concomitant increase in bone citrate concentration with a peak value lagging behind that in the blood. The second effect, owing to the addition of vitamin D, had an observed peak value for serum citrate 16 days after the beginning of rehabilitation, preceded by a
maximum bone citrate concentration observed after only 2 days. These two effects can be observed independently in groups 2 and 3 (Tables 2 and 3) and together in group 4 (Table 4).

Our tentative conclusions are, therefore, that when conditions of severe calcium and vitamin D deficiencies are ameliorated by the provision of the vitamin alone, the effect is to increase the mobilization of calcium from the skeleton to the blood. This transference of calcium is associated with a progressive increase followed by a decline of serum citrate and a transient increase in bone citrate concentration which precedes the maximum increase in blood citrate. We suggest, therefore, that in these circumstances the synthesis of citrate occurs primarily in the bone and, if not the cause, it is certainly not the result of the rise in serum citrate. Replenishment of the diet with calcium alone resulted in a rapid and sustained rise in serum calcium from dietary sources, with a rapid but transient increase in serum citrate followed by a sustained rise in bone citrate concentration with a peak value after 7 days. In these circumstances the rise in concentration of the citrate in bone appears to be secondary to that in the serum.

Rehabilitation with both calcium and vitamin D produces a combination of both effects. In earlier experiments (Hartles and Leaver, 1961a,b) it was shown that the accumulation of citrate in bone was directly governed by vitamin D when the diet was low in calcium. In the absence of added vitamin D, however, a sufficiency of dietary calcium enabled the concentration of bone citrate to approach the normal value. This finding was in keeping with the results of Nicolaysen and EEG-Larson (1956). It is, however, a confusing situation which cannot apparently be reconciled with the hypothesis of Neuman and Neuman (1958) which attempts to correlate the action of vitamin D and parathyroid hormone with the synthesis of citrate in bone. If, however, as we suggest, the citrate accumulating in bone as a result of adding calcium to a diet deficient in both calcium and the vitamin, is not produced in the bone but arises secondarily, then this goes some way towards reconciling our results with the Neuman's hypothesis. Briefly our understanding of their hypothesis is as follows. Vitamin D and parathyroid hormone influence different co-factors in carbohydrate metabolism; the vitamin stimulates reactions requiring NAD or co-carboxylase, the hormone inhibits oxidative reactions requiring NADP. Vitamin D will therefore tend to encourage the production of citrate, parathyroid hormone will hinder its further oxidation and will also encourage glycolysis and the production of pyruvate by inhibiting the NADP-dependent pentose-oxidation shunt. The hormone alone cannot stimulate citrate production whereas vitamin D alone can. Together they will exert the maximum effect. Thus, if this hypothesis is valid, those circumstances in which the production of the hormone is stimulated when vitamin D is present should lead to increased synthesis of bone citrate. Conversely, lessening of hormonal activity in the absence of the vitamin should result in minimal synthesis of citrate.

It is not unreasonable to assume that in the animals maintained on the low-calcium diets the parathyroids would in general be stimulated to a greater degree than in animals receiving an adequate supply of dietary calcium. We reported earlier
(Hartles and Leaver, 1961a,b) that in the presence of vitamin D the bone of rats contained more citrate when they were maintained from weaning on diets low in calcium, than when the calcium intake was normal. These results are in accord with the Neumans' hypothesis. The anomalous result was that in the absence of the vitamin less citrate was found in the bone on a low-calcium diet (parathyroid stimulated) than on a normal calcium intake (parathyroid less stimulated). Our present results suggest that in these latter circumstances the bone citrate is not produced in situ but is secondarily acquired from the body fluids in the process of mineralization. This observation helps to resolve the above anomaly and supports the view that the synthesis of bone citrate in situ may be governed by both the parathyroid hormone and vitamin D. It also emphasises that the total citrate content of a bone is not necessarily a reflection of the synthesis within the bone.

In putting forward this concept we are aware that we are raising other problems. Inherent in our suggestion are the ideas that the extra-skeletal synthesis of citrate is not so dependent on the presence of vitamin D and that its lack is less important when the animal's calcium nutrition is adequate. These notions gain some support from the observations of De Luca et al. (1961), who found different responses in the formation of citrate by bone and other tissues as a result of changes in dietary B-vitamins. They suggest the possibility that the synthesis of bone citrate may be subject to metabolic controls which differ from those operating in the other tissues. We hope to gather further information on this vexed problem by studying the rehabilitation of rats maintained on diets low in phosphorus and vitamin D but adequate in calcium.

Acknowledgements—The authors thank Mr. R. P. Williams for his expert care of the animals, Miss G. A. Rixom for technical assistance in the analytical work, Messrs. Tate and Lyle Ltd. for the gifts of icing sugar, and Messrs. J. Bibby & Sons Ltd. for the gifts of ground nut oil.

REFERENCES


