THE INFLUENCE OF TWO DIPHOSPHONATES ON CALCIUM METABOLISM IN THE RAT

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SUMMARY

1. The effects of two diphosphonates, disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) and disodium dichloromethylene diphosphonate (Cl₂MDP) on several variables of calcium metabolism have been measured in the intact rat using the model of Aubert & Milhaud (1960).

2. The animals were bred from weaning on a diet containing 1·3% calcium. At the age of 54 days they were switched to a diet with 0·5% calcium. Some of the animals were also given daily injections of one of the diphosphonates from the day the intake was decreased. The doses of the compounds were 0·01–10 mg of P kg body wt.⁻¹ day⁻¹.

3. Cl₂MDP caused a decrease in the rates of bone formation ($V_{o+}$) and bone resorption ($V_{o-}$), and these effects were proportional to the logarithm of the dose of the compound. There was a small increase in the retention of calcium due to a decrease in the endogenous faecal calcium.

4. EHDP in doses of up to 1 mg of P kg⁻¹ day⁻¹ decreased bone resorption but had little effect on bone formation. The retention of calcium increased due to an increased absorption of dietary calcium and a decreased endogenous faecal calcium. EHDP at a dose of 10 mg of P kg⁻¹ day⁻¹ caused a further fall in $V_{o-}$ but also a great decrease in $V_{o+}$ which was larger than the fall in $V_{o-}$. The net absorption of calcium was diminished, the urinary excretion of calcium was increased, the retention of calcium was greatly diminished and there was a greatly diminished ash content in the femur. Thus, for equal doses of up to 1 mg of P kg⁻¹ day⁻¹, Cl₂MDP was more potent in decreasing bone turnover than EHDP, but the two compounds caused a similar increase in the retention of calcium. At high doses EHDP inhibited the mineralization of epiphyseal cartilage.

5. These results are discussed in relation to the possible clinical applications of the diphosphonates.

Key words: diphosphonates, calcium metabolism, bone formation, bone resorption.

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Previous studies have shown that pyrophosphate diminished both the rate of formation and the rate of dissolution of apatite crystals in vitro (Fleisch, Russell & Straumann, 1966). It was suggested that the pyrophosphate present in blood, bones and teeth might have similar effects in vivo (Fleisch, Russell, 1970). However, although exogenous pyrophosphate prevented soft tissue calcification induced by various means (Schibler & Fleisch, 1966; Schibler, Russell & Fleisch, 1968), it had no effect on either the deposition (Irving, Schibler & Fleisch, 1966) or the removal of the calcium phosphate of bone (Russell, Mühlbauer, Bisaz, Williams & Fleisch, 1970). The lack of effect on bone might be due to enzymic destruction of the pyrophosphate. Compounds with similar structure to pyrophosphate, the diphosphonates, which contain the P–C–P bond and are resistant to enzymic destruction, resemble pyrophosphate in their ability to diminish the rates of formation and dissolution of apatite crystals in vitro (Fleisch, Russell, Bisaz, Casey & Mühlbauer, 1968; Francis, 1969; Francis, Russell & Fleisch, 1969; Fleisch, Russell & Francis, 1969a; Fleisch, Russell, Bisaz, Mühlbauer & Williams, 1970; Russell et al., 1970). The diphosphonates are also effective in vivo. The effects, which have been demonstrated with several diphosphonates, include an inhibition of soft-tissue calcification in animals (Fleisch et al., 1968; Francis et al., 1969; Fleisch et al., 1970) and in man (Bassett, Donath, Macagno, Preisig, Fleisch & Francis, 1969), an inhibition of urinary calcium-containing stones in rats (Fraser, Russell, Pohler, Robertson & Fleisch, 1972), an inhibition of bone calcification (King, Francis & Michael, 1971; Jowsey, Holley & Linman, 1970; R. Schenk, W. A. Merz, H. Fleisch, R Mühlbauer & R. G. G. Russell, unpublished observations), an inhibition of the effect of parathyroid hormone on bone resorption in tissue culture (Russell et al., 1970), an inhibition of the hypercalcemia due to parathyroid hormone in thyroparathyroidectomized rats (Fleisch et al., 1969a; Russell et al., 1970), and a decrease of the osteoporosis due to immobilization in rats (Fleisch, Russell, Simpson & Mühlbauer, 1969b; Michael, King & Francis, 1971; Mühlbauer, Russell, Williams & Fleisch, 1971).

The aims of the present study was to examine the effects of two diphosphonates, disodium dichloromethylene diphosphonate (Cl₂MDP) and disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP), on several variables of calcium metabolism measured simultaneously in intact rats deprived of calcium from the time they were given the diphosphonates.

The experiments were made in growing rats since their calcium metabolism has been studied in detail (Aubert & Milhaud, 1960; Milhaud, Remagen, Gomes de Matos & Aubert, 1960; Richelle, 1967; Bronner, 1967; Cohn, Teree & Gusmano, 1968). The 61-day-old animal was chosen since this is the age when the rate of bone formation is maximal (Richelle, Onkelinx & Aubert, 1966). In rats bred on a 1·3% calcium diet, as our rats were, bone resorption rate measured as $V_{o-}$ is small (Bronner & Aubert, 1965), therefore it was necessary to increase $V_{o-}$ by a short-term reduction in calcium intake (Bronner & Aubert, 1965; Cohn et al., 1968).

The study was performed by the simultaneous measurement of the intake and excretion of stable calcium and the measurement of $^{45}$Ca in plasma, urine and faeces after a single intravenous injection of $^{45}$Ca. The measurements of stable calcium allow calculation of absorption of calcium from the intestine. The radioactivity data have been analysed according to the model of Aubert & Milhaud (1960) and Richelle (1967), which gives an estimate of bone formation rate ($V_{o+}$). The bone resorption rate can then be calculated as the difference between $V_{o+}$ and the retention of stable calcium. This model has been used to study the effect of several treatments on these variables, including parathyroid hormone (Aubert, Cherian, Moukhtar & Milhaud, 1964), calcitonin (Milhaud & Moukhtar, 1966; Milhaud, Moukhtar, Cherian & Pérault, 1966),
vitamin D (Hurwitz, Stacey & Bronner, 1969), phosphate deprivation (Milhaud et al., 1960; Hurwitz et al., 1969) and variation in calcium intake (Bronner & Aubert, 1965; Cohn et al., 1968).

MATERIALS AND METHODS

Experimental design

Female Wistar rats from our own breeding colony were given from weaning a commercial chow (No. 194, Nafag, Gossau) containing 1.3% Ca and 1.0% P (dry weight). At the age of 54±1 days they were weighed and put into single metabolic cages. From that time until the end of the experiment 10 days later they received a commercial chow (No. 195 OPC, Nafag, Gossau) which contained 0.2% Ca and 0.3% P, to which was added Ca₃(PO₄)₂ and calcium gluconate to give a final content of 0.5% Ca and 0.35% P.

To minimize the variation in food intake between the animals, the following procedure was used: on the first day of the experiment all animals were given 14 g of dry food. This amount was reduced daily by 1 g until none of the animals left more than 0.5 g of their daily ration. This amount of food, which was usually 12 g, was given daily to all animals for the remainder of the experiments. Any food which remained in the food cups or had fallen through the bottom of the cage was weighed and subtracted from the daily ration.

The animals were put into the metabolic cages and allocated at random to groups which were given each morning for 10 days subcutaneous injections of 0, 0.01, 0.1, 1.0 or 10.0 mg of P/kg body wt. of one of the diphosphonates. The diphosphonates were dissolved in water and the solution was adjusted to pH 7.4. On the 6th day urine was collected during 24 h into tubes, through a device for the separation of urine and faeces. On the 7th day the animals were given a single intravenous injection of about 30 µCi of ⁴⁵Ca (Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland) pH 6-7, ⁴⁵CaCl₂, (specific radioactivity 30 µCi/mg of Ca) at the base of the tail. The ⁴⁵Ca was injected about 5 min after the injection of diphosphonate. An equal volume of ⁴⁵CaCl₂, diluted in 500 ml of water, was taken as the standard for the counting of ⁴⁵Ca radioactivity in the samples.

Techniques

The following procedures were carried out according to the techniques described by Milhaud et al. (1960) and in more detail by Richelle (1967).

Collection of samples. Blood samples were taken from the tip of the tail into heparinized micro-haematocrit tubes (No. A-2930, Clay-Adams) 2, 4, 6, 24, 48 and 72 h after the injection of ⁴⁵Ca.

Urine was collected on a sheet of filter paper (P-free, No. 512, Schleicher & Schuell) which was supported on a net of nylon underneath the metabolic cages. The collection of urine started when the ⁴⁵Ca was injected, and continued for 72 h.

To time the collection of faeces, the rats were given two doses of Carmine Red [0.5 ml of 6% (w/v) in water] through an intragastric tube. The first dose was given 5 h after the injection of ⁴⁵Ca and the second dose 72 h after the first. The faeces stained with the first marker were collected but those stained with the second marker were not.

When the collection of faeces was complete, the rats were anaesthetized with ether, and blood was taken from the heart for the measurement of calcium and phosphate. The rats were then killed and the right femur was taken out.
Preparation of samples. Blood samples were centrifuged in the micro-haematocrit tubes. The tubes were cut and the part containing the plasma was weighed. The plasma was blown from the tube on to a planchette and the empty tube was weighed so that the weight of plasma on the planchette could be calculated. The plasma of the planchette was diluted with a few drops of detergent (Mucapur-M, Müller & Krempel, Bülach, Switzerland), the planchette was filled with water and the sample left to dry at room temperature.

After removal of the faeces the filter papers contained urine and spilled food, which was probably contaminated with urine. The food was removed from the paper, dried for 12 h at 105°C, weighed and put back on the paper. The papers were dried for 12 h at 105°C and they were ashed together with the dried spilled food at 750°C for 12 h. The ash was dissolved in 10 ml of 1 M-HCl at about 60°C and made up to 25 ml with water. For the measurement of \(^{45}\text{Ca}\), 250 µl of the solution was mixed with a solution of CaCO\(_3\) (Merck, purum, 2.5 g/l in water) so that the mixture contained a total of 2.5 mg of Ca including the calcium originally present. The calcium in this mixture was precipitated by the addition of 2 ml of ammonium oxalate (Merck, p.a., 3% in water) and by adding NH\(_4\)OH (10%) to give a pH of 6–8. To ensure complete precipitation the mixture was kept at 60°C for 1 h and then left overnight at room temperature. The precipitate was collected by filtering through discs of filter paper (No. 576, Schleicher & Schuell) of the same size as the planchettes.

The faeces were dried and ashed in the same way as the filter papers containing the urine. The ash was dissolved in 20 ml of 1 M-HCl at 60°C and the solution was diluted to 250 ml with water. Samples (250 µl) of the final solution were prepared for the measurement of \(^{45}\text{Ca}\) by the technique described above for the solutions of the ashed filter papers. The calcium and phosphorus contents of the final solutions were also measured.

The femora were cleaned and their lengths measured. They were then successively dried for 12 h at 105°C, weighted, defatted with an excess of ethanol–trichlorethylene (1:1 v/v) and ashed for 12 h at 700°C. The ash was weighed and dissolved in 5 ml of 1 M-HCl at 60°C and the solution was made up to 250 ml with water. The Ca and P contents of the solutions were measured. Samples (250 µl) of the solutions were prepared for the measurement of \(^{45}\text{Ca}\) in the same way as the solutions of ashed faeces.

Samples of the standard solution of \(^{45}\text{CaCl}_2\) were treated in the same way as samples of plasma and samples of the solutions of ashed filter paper.

Chemical methods. Calcium was measured with an atomic absorption spectrophotometer (Perkin-Elmer 290B) after dilution of the samples with lanthanum chloride (1% in 5% HCl, v/v). Phosphorus was measured according to the method of Chen, Toribara & Warner (1956). Hydroxyproline was measured in hydrolysed urine with the technique of Prockop & Udenfriend (1960) as modified by Kivirikko, Laitinen & Prockop (1967).

Measurement of \(^{45}\text{Ca}\). The \(^{45}\text{Ca}\) content of the dried plasma and the precipitates of calcium oxalate from faeces, urine and bone was measured in a methane-flow radioactivity counter (Friesecke & Högpfner, Erlangen-Bruck, Germany). The plasma was counted without a window, and the precipitates with a window, and the counting efficiency was 30% and 15%, respectively. Samples of the standard solution of \(^{45}\text{CaCl}_2\) prepared like samples of plasma and urine were counted at the same time as the samples of plasma and calcium oxalate precipitates.

Measured variables of calcium metabolism

Balance study. The following variables can be calculated directly from the measurements
of calcium in the food and faeces: \( V_I \), intake of calcium; \( V_F \), faecal output of calcium; \( V_{na} \), net absorption. \( V_{na} \) is the difference between the intake of calcium and the faecal output of calcium; \( V_{na} = V_I - V_F \). It corresponds to the \( S_i \) of Aubert & Bronner (1965). \( V_u \), urinary output of calcium. It is calculated as the total \(^{45}\text{Ca}\) in urine divided by the integral of the serum specific activity during the 72 h.

From these it is possible to calculate \( V_s \), calcium retention by the body. This is the difference between net absorption (\( V_{na} \)) and urinary excretion (\( V_u \)). \( V_s \) corresponds to the \( \Delta \) of Milhaud et al. (1960). It is assumed that this calcium is retained in bone.

**Fluxes of calcium into and out of the intestine.** The endogenous faecal calcium (\( V_f \)) was calculated as the total \(^{45}\text{Ca}\) in the faeces divided by the integral of the serum specific activity. The absorption of dietary calcium (\( V_{ad} \)) was calculated as the difference between the calcium intake and excretion of non-radioactive calcium in the faeces [\( V_{ad} = V_I - (V_F - V_f) \)]. \( V_{s1} \) is the rate of secretion of calcium into the intestine, calculated on the assumption that calcium is secreted below the sites of absorption so that all secreted calcium appears in the faeces. Thus \( V_{s1} = V_f \). \( V_{s2} \) is the rate of secretion of calcium into the intestine, calculated on the assumption that all the calcium is secreted into the proximal small intestine and is then reabsorbed to the same extent that dietary calcium is absorbed. Thus:

\[
\frac{V_f}{V_{s2}} = \frac{V_F - V_I}{V_I} \quad \text{or} \quad V_{s2} = \frac{V_I}{V_F - V_I} V_f
\]

For further discussion of the reabsorption of secreted calcium see Aubert, Bronner & Richelle (1963), Bronner (1964) and Marshall (1969).

**Fluxes of calcium into and out of bone.** The changes in the specific radioactivity of the plasma calcium were analysed according to the open two-compartment model described by Aubert & Milhaud (1960). The two exponential equation which best fitted the six values for serum specific activity was obtained by the standard technique of 'curve peeling'. The following variables can be calculated on the basis of this model (Fig. 1):

\[\begin{align*}
E_1, \text{ the mass of calcium with which the injected } ^{45}\text{Ca} \text{ has mixed in 2 h;} \\
E_2, \text{ the slowly exchangeable pool. This is the mass of calcium (or pool) which has exchanged with } E_1 \text{ between 2 and 72 h. } \nu_e, \text{ the flux of calcium between } E_1 \text{ and } E_2; \\
\nu_{0+}, \text{ the flux of calcium into bone. This is calculated as the loss of calcium from } E_1 \text{ which is not accounted for by the excretion of calcium in urine (} \nu_u \text{) or faeces (} \nu_f \text{). It is taken as an estimate of the rate of bone formation (see the}
\end{align*}\]
Discussion section). $V_{o-}$, the flux of calcium out of bone. It is calculated as the difference between the rate of entry of calcium into bone ($V_{o+}$) and the rate of calcium retention in the body, i.e. in the bone ($V_o$). The $V_{o-}$ is taken as an estimate of the rate of bone resorption (see the Discussion section).

The fluxes are calculated in mg/day and the exchangeable masses ('pools') in mg. These variables were calculated on a CDC 3600 computer with the program described by Richelle (1967).

**RESULTS**

**Effects of Cl₂MDP**

The major changes are shown in Fig. 2; the complete results are given in Table 1. The larger doses of this compound (1 and 10 mg of P kg⁻¹ day⁻¹) caused a small increase in the net absorption of calcium ($V_{na}$) and in the retention of calcium ($V_o$). There were no significant changes in the urinary excretion of calcium ($V_u$).

There was no significant change in the absorption of dietary calcium ($V_{ad}$). But there was a decrease in the rate of secretion of calcium into the intestine on whichever assumption this was
Table 1. Influence of various doses of ClzMDP on several variables of calcium metabolism in rats aged 61 days. ClzMDP was administered daily, subcutaneously, from the age of 54 days. The animals were given a diet with 1.3% calcium until they were aged 54 days and then a diet with 0.5% calcium until they were killed. The values are the mean ± SEM. The significance of the difference from the control group by the t-test is indicated as * = P<0.05; ** = P<0.01; *** = P<0.001.

<table>
<thead>
<tr>
<th>Fluxes (mg/day)</th>
<th>0 (control)</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_1$</td>
<td>54.49±0.75</td>
<td>52.23±0.72*</td>
<td>51.79±1.30</td>
<td>52.74±0.78</td>
<td>52.20±0.92</td>
</tr>
<tr>
<td>$V_2$</td>
<td>25.10±0.82</td>
<td>23.96±1.64</td>
<td>21.24±1.18*</td>
<td>18.56±0.90***</td>
<td>17.49±1.49***</td>
</tr>
<tr>
<td>$V_{na}$</td>
<td>28.89±0.74</td>
<td>28.27±1.64</td>
<td>30.55±0.87</td>
<td>33.97±0.85***</td>
<td>34.71±1.37***</td>
</tr>
<tr>
<td>$V_a$</td>
<td>2.75±0.66</td>
<td>2.03±0.23</td>
<td>1.89±0.43</td>
<td>1.79±0.43</td>
<td>2.90±0.36</td>
</tr>
<tr>
<td>$V_0$</td>
<td>26.64±0.78</td>
<td>25.69±1.90</td>
<td>28.67±1.15</td>
<td>32.39±1.17***</td>
<td>31.82±1.62*</td>
</tr>
<tr>
<td>$V_{ad}$</td>
<td>35.89±0.74</td>
<td>34.05±1.48</td>
<td>33.49±1.14</td>
<td>37.21±0.92</td>
<td>37.22±1.19</td>
</tr>
<tr>
<td>$V_{r} = V_{st}$</td>
<td>6.50±0.49</td>
<td>5.78±0.08</td>
<td>2.94±0.70***</td>
<td>3.03±0.29***</td>
<td>2.51±0.54***</td>
</tr>
<tr>
<td>$V_{s2}$</td>
<td>19.46±1.80</td>
<td>16.74±2.56</td>
<td>10.78±2.49*</td>
<td>11.07±1.16*</td>
<td>8.55±1.58**</td>
</tr>
<tr>
<td>$V_{0,e}$</td>
<td>71.61±4.30</td>
<td>62.69±8.29</td>
<td>53.96±6.77*</td>
<td>50.03±3.77***</td>
<td>35.39±3.41***</td>
</tr>
<tr>
<td>$V_{0,c}$</td>
<td>44.97±4.41</td>
<td>37.00±8.61</td>
<td>25.29±7.30*</td>
<td>16.81±4.45***</td>
<td>3.57±3.95***</td>
</tr>
<tr>
<td>$V_e$</td>
<td>107.33±9.49</td>
<td>103.47±20.13</td>
<td>79.69±11.93</td>
<td>73.42±9.84*</td>
<td>75.43±14.26</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pools (mg)</th>
<th>0 (control)</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_1$</td>
<td>25.48±2.18</td>
<td>23.97±4.22</td>
<td>17.51±1.40**</td>
<td>17.84±1.55**</td>
<td>17.48±2.35*</td>
</tr>
<tr>
<td>$E_2$</td>
<td>73.83±8.43</td>
<td>66.49±12.19</td>
<td>58.95±11.44</td>
<td>42.25±4.46**</td>
<td>39.44±5.55**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum concentration (mg/100 ml)</th>
<th>[Ca₄]</th>
<th>[P₄]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{Ca}_4]$</td>
<td>10.61±0.18</td>
<td>10.40±0.12</td>
</tr>
<tr>
<td>$[\text{P}_4]$</td>
<td>7.08±0.17</td>
<td>7.04±0.18</td>
</tr>
</tbody>
</table>
calculated \((V_{s1} \text{ or } V_{s2})\). This decrease in secreted calcium, which was also present after a dose of \(0.1 \text{ mg of P kg}^{-1} \text{ day}^{-1}\), explained most of the increase in the net absorption of calcium. \(\text{Cl}_2\text{MDP}\) diminished the rates of bone formation \((V_{0+})\) and bone destruction \((V_{0-})\) at doses of \(0.1 \text{ mg kg}^{-1} \text{ day}^{-1}\) or greater. Both rates were inversely proportional to the logarithm of the dose of the compound.

**Table 2.** Influence of various doses of \(\text{Cl}_2\text{MDP}\) and EHDP on the urinary excretion of hydroxyproline \((\text{mg/24 h})\) in rats aged 60 days. \(\text{Cl}_2\text{MDP}\) or EHDP were administered daily, subcutaneously, from the age of 54 days. The animals had a diet with 1.3% calcium until the age of 54 days, then with 0.5% calcium until they were killed. 

\(P\) values are indicated as in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Cl}_2\text{MDP})</td>
<td>504.05</td>
<td>518.62</td>
<td>453.57</td>
<td>398.40</td>
<td>341.28</td>
</tr>
<tr>
<td></td>
<td>(\pm 24.17)</td>
<td>(\pm 54.90)</td>
<td>(\pm 47.01)</td>
<td>(\pm 14.71^{**})</td>
<td>(\pm 36.12^{***})</td>
</tr>
<tr>
<td>(\text{EHDP})</td>
<td>432.50</td>
<td>389.00</td>
<td>458.14</td>
<td>317.75</td>
<td>256.62</td>
</tr>
<tr>
<td></td>
<td>(\pm 31.14)</td>
<td>(\pm 44.34)</td>
<td>(\pm 78.04)</td>
<td>(\pm 25.93^{**})</td>
<td>(\pm 29.45^{***})</td>
</tr>
</tbody>
</table>

**Table 3.** Influence of various doses of \(\text{Cl}_2\text{MDP}\) and EHDP on the dry weight and the ash content of the femora of rats aged 65 days. The diphosphonates were given daily from the age of 54 days. All rats were given a diet with 1.3% calcium until 54 days of age and then a diet with 0.5% calcium. The values for dry weight and ash content in a group of twelve rats aged 54 days was 281.8 \(\pm 6.1\) mg and 161.5 \(\pm 3.6\) mg, respectively. \(P\) values are indicated as in Table 1.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose of the diphosphonate (mg of P kg(^{-1}) day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>(\text{Cl}_2\text{MDP})</td>
<td>Dry weight (mg)</td>
</tr>
<tr>
<td></td>
<td>Ash weight (mg)</td>
</tr>
<tr>
<td>(\text{EHDP})</td>
<td>Dry weight (mg)</td>
</tr>
<tr>
<td></td>
<td>Ash weight (mg)</td>
</tr>
</tbody>
</table>

The flux of calcium \((V_e)\) between the exchangeable pools \((E_1 \text{ and } E_2)\) was less with the two larger doses of the compound. But the variation in \(V_e\) in any group was large so that the relation between \(V_e\) and the dose of the compound could not be defined accurately. There was a significant decrease in both exchangeable pools \((E_1 \text{ and } E_2)\) with the two largest doses of the compound (1 and 10 mg of P kg\(^{-1}\) day\(^{-1}\)).

The compound had no significant effect on the plasma calcium concentration, but caused a decrease in plasma phosphorus concentration which was greatest with the largest dose of the
Effect of diphosphonates on calcium metabolism

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compound. Table 2 shows that the urinary excretion of hydroxyproline measured after 6 days of calcium deprivation was less in the rats treated with Cl$_2$MDP, and this decrease was greatest with the largest doses of Cl$_2$MDP. Table 3 shows that the weight of ash in the femur was increased in the animals given the two larger doses of the compound.

Effects of EHDP

The two smaller doses of EHDP had no significant effect on any of the measured variables of calcium metabolism (Fig. 3, Table 4). The two larger doses (1·0 and 10·0 mg of P kg$^{-1}$ day$^{-1}$) changed several of the variables. However, the magnitude and the direction of these effects were different with the two doses.

![Fig. 3. Influence of various doses of EHDP on calcium metabolism in rats aged 61 days. EHDP was administered daily, subcutaneously, from the age of 54 days. The animals were given a diet with 1·3% calcium until 54 days of age and then a diet with 0·5% calcium until they were killed. The values shown are the mean ±1 SEM for each treatment group.](image)

Effects of a dose of 1 mg of P kg$^{-1}$ day$^{-1}$ of EHDP

This dose of EHDP increased the net absorption of calcium ($V_{na}$) and the retention of calcium ($V_{r}$). There was no change in urinary excretion of calcium ($V_{u}$).

The increase in net absorption was due both to an increase in absorption of dietary calcium ($V_{ad}$) and a decrease in endogenous faecal calcium ($V_{f}$). While the rate of secretion of calcium into the intestine calculated as $V_{s1} = V_{f}$ did change, the rate of secretion of calcium into the
Table 4. Influence of various doses of EHDP on some variables of calcium metabolism in rats aged 61 days. EHDP was administered daily, subcutaneously, from the age of 54 days. The animals were given a diet with 1.3% calcium until they were aged 54 days and then a diet with 0.5% calcium until they were killed. *P values are indicated as in Table 1.

<table>
<thead>
<tr>
<th>Dose (mg of P kg(^{-1}) day(^{-1}))</th>
<th>0 (control)</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluxes (mg/day)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(V_1)</td>
<td>48.47 ± 0.96</td>
<td>48.60 ± 0.78</td>
<td>48.37 ± 0.67</td>
<td>48.29 ± 0.71</td>
<td>47.58 ± 1.28</td>
</tr>
<tr>
<td>(V_F)</td>
<td>25.81 ± 1.22</td>
<td>26.21 ± 1.24</td>
<td>23.83 ± 2.18</td>
<td>18.58 ± 1.29***</td>
<td>33.61 ± 2.50**</td>
</tr>
<tr>
<td>(V_{na})</td>
<td>22.66 ± 0.92</td>
<td>22.39 ± 1.56</td>
<td>24.54 ± 2.72</td>
<td>29.71 ± 1.15***</td>
<td>15.96 ± 2.09**</td>
</tr>
<tr>
<td>(V_a)</td>
<td>1.04 ± 0.06</td>
<td>1.12 ± 0.16</td>
<td>1.45 ± 0.43</td>
<td>0.98 ± 0.15</td>
<td>4.28 ± 0.83***</td>
</tr>
<tr>
<td>(V_0)</td>
<td>21.61 ± 0.99</td>
<td>21.27 ± 1.51</td>
<td>23.10 ± 2.62</td>
<td>28.74 ± 1.23***</td>
<td>9.15 ± 3.28**</td>
</tr>
<tr>
<td>(V_{ad})</td>
<td>29.26 ± 0.74</td>
<td>29.56 ± 1.45</td>
<td>30.75 ± 2.22</td>
<td>34.40 ± 0.95***</td>
<td>21.95 ± 2.42**</td>
</tr>
<tr>
<td>(V_t = V_s)</td>
<td>6.61 ± 0.43</td>
<td>7.17 ± 0.37</td>
<td>6.21 ± 0.69</td>
<td>4.68 ± 0.26***</td>
<td>7.62 ± 0.45</td>
</tr>
<tr>
<td>(V_{s2})</td>
<td>16.64 ± 0.83</td>
<td>18.76 ± 1.59</td>
<td>17.19 ± 1.85</td>
<td>16.65 ± 0.83</td>
<td>14.74 ± 1.74</td>
</tr>
<tr>
<td>(V_{0-})</td>
<td>61.18 ± 1.82</td>
<td>64.27 ± 4.15</td>
<td>58.04 ± 2.40</td>
<td>57.30 ± 2.07</td>
<td>19.46 ± 1.06***</td>
</tr>
<tr>
<td>(V_{0-})</td>
<td>39.56 ± 2.30</td>
<td>43.00 ± 3.73</td>
<td>34.94 ± 2.92</td>
<td>28.56 ± 1.75***</td>
<td>10.32 ± 2.88***</td>
</tr>
<tr>
<td>(V_e)</td>
<td>89.75 ± 3.67</td>
<td>88.08 ± 7.23</td>
<td>91.44 ± 8.81</td>
<td>81.46 ± 3.26</td>
<td>40.10 ± 2.05***</td>
</tr>
<tr>
<td>Pools (mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E_1)</td>
<td>22.39 ± 1.12</td>
<td>23.01 ± 2.25</td>
<td>21.16 ± 0.80</td>
<td>20.60 ± 1.07</td>
<td>17.94 ± 0.89***</td>
</tr>
<tr>
<td>(E_2)</td>
<td>68.75 ± 3.73</td>
<td>64.96 ± 7.99</td>
<td>59.01 ± 4.33</td>
<td>61.25 ± 3.61</td>
<td>22.19 ± 1.50***</td>
</tr>
<tr>
<td>Serum concentration (mg/100 ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Ca](^+)</td>
<td>10.42 ± 0.13</td>
<td>10.66 ± 0.22</td>
<td>10.79 ± 0.20</td>
<td>10.40 ± 0.29</td>
<td>11.35 ± 0.30*</td>
</tr>
<tr>
<td>[P(^{31})]</td>
<td>8.15 ± 0.18</td>
<td>8.28 ± 0.27</td>
<td>8.12 ± 0.42</td>
<td>8.02 ± 0.18</td>
<td>7.65 ± 0.55</td>
</tr>
</tbody>
</table>
Effect of diprophonates on calcium metabolism

intestine calculated as $V_{s2}$ did not. Thus, if it is assumed that secreted calcium is absorbed like dietary calcium, the decrease in $V_t$ can be explained by the increased efficiency of calcium absorption.

This dose of EHDP had no significant effect on the rate of bone formation ($V_{0+}$) and the decrease in the rate of bone resorption ($V_{0-}$) was much less than that observed with the corresponding dose of Cl$_2$MDP. In spite of these differences in the effects of the two compounds on bone turnover, it should be emphasized that at this dose (1 mg of P kg$^{-1}$ day$^{-1}$) the two diphosphonates had similar effects on the retention of calcium ($V_o$). There were no significant changes in the flux of calcium between the exchangeable pools ($V_e$) or in the size of either of these two pools ($E_1$ and $E_2$).

There were no significant effects on the serum calcium or phosphorus concentrations.

This dose of EHDP diminished the hydroxyproline excretion in the urine (Table 2) but had no effect on the ash content of the bones (Table 3).

Effects of a dose of 10 mg of P kg$^{-1}$ day$^{-1}$ of EHDP

This large dose of EHDP had several effects which were not seen with Cl$_2$MDP. There was a large decrease in the net absorption of calcium ($V_{na}$) but an even greater decrease in the retention of calcium ($V_o$) and there was an increased urinary excretion of calcium ($V_e$).

The absorption of dietary calcium ($V_{s2}$) decreased while the endogenous faecal calcium ($V_f$) increased. Thus, if the secreted calcium is assumed to be entirely secreted in the faeces ($V_{s1} = V_f$), there was an increased secretion of calcium into the intestine. On the other hand, if it is assumed that calcium is secreted above the site of absorption and absorbed like dietary calcium ($V_{s2}$), there was no change in the rate of secretion of calcium into the intestine. Thus, on the basis of the second hypothesis concerning secreted calcium ($V_{s2}$), the increase in $V_t$ can be accounted for by the decreased absorption of dietary, and thus of secreted, calcium.

Bone formation rate ($V_{0+}$), which was not influenced by the smaller doses of EHDP, was greatly diminished after this large dose. The rate of bone destruction ($V_{0-}$) was also less than with the smaller doses. However, the decrease in bone formation rate ($V_{0+}$) was greater than the decrease of bone resorption rate ($V_{0-}$), whereas Cl$_2$MDP had about equal effects on the two rates. This large dose of EHDP also decreased the flux of calcium between the exchangeable pools of calcium ($V_e$) and the size of the rapidly and slowly exchangeable pools of calcium ($E_1$ and $E_2$).

The serum calcium concentration was increased after the large dose of EHDP but there was a small decrease in the serum phosphorus.

There was a greatly diminished urinary excretion of hydroxyproline in these rats (Table 2). The ash content of the femur also was less in the rats given this dose of EHDP (Table 3).

DISCUSSION

Plasma calcium

The changes in plasma calcium were small in relation to the large changes in $V_{0+}$ and $V_{0-}$. In Cl$_2$MDP-treated rats the kinetic parameters were diminished to about the same extent as after parathyroidectomy (Aubert et al., 1964) and, despite this, the plasma calcium concentration was decreased only very slightly. Thus, the plasma calcium concentration is not directly related to absolute rates of bone resorption and the rat is able to maintain its plasma calcium
concentration even when its ability to alter bone resorption is impaired. Also with EHDP, even when doses large enough to inhibit mineralization of bone matrix were given, the change in plasma calcium was small. Thus, the rat also seems able to regulate plasma calcium when bone mineralization is interfered with. This suggests that other organs such as gut or kidneys, as emphasized by Nordin & Peacock (1969), must play an important role.

**Plasma phosphate**

There was a progressive decrease in the plasma phosphate concentration with increasing doses of Cl$_2$MDP. This was not accompanied by a change in urinary phosphate output so that a marked decrease in renal reabsorption of phosphate must have occurred. This suggests that a stimulation of secretion of parathyroid hormone (PTH) has taken place.

$V_{0+}$

$V_{0+}$ provides a measure of the flux of calcium into the bone. It correlates with the rate of bone formation (matrix plus mineral) as long as there is no dissociation between rates of matrix formation and matrix mineralization. With Cl$_2$MDP, where this seems to be the case since unmineralized matrix is not seen by histological techniques (R. Schenk, W. A. Merz, H. Fleisch, R. Mühlbauer & R. G. G. Russell, unpublished observations), a decrease in $V_{0+}$ probably represents a decrease in matrix and mineral formation rate. This effect could well be secondary to the decrease in resorption. In contrast, with large doses of EHDP (10 mg of P kg$^{-1}$ day$^{-1}$), there is an additional direct impairment of matrix mineralization since studies of bone morphology showed a widened, non-mineralized epiphyseal plate and large osteoid seams (R. Schenk, W. A. Merz, H. Fleisch, R. Mühlbauer & R. G. G. Russell, unpublished observations). Thus, in this case the decrease in $V_{0+}$ can indicate a decrease in matrix mineralization as well as a decrease in matrix and mineral formation.

**Intestinal calcium absorption**

The decrease in intestinal absorption with EHDP at 10 mg of P kg$^{-1}$ day$^{-1}$ could be due to a direct effect of EHDP on the intestine or could be secondary to the effect on mineralization. Various studies suggest that the first possibility is unlikely (Morgan, Bonjour, Gasser, O’Brien & Fleisch, 1971; J.-P. Bonjour, R. G. G. Russell, D. B. Morgan & H. Fleisch, unpublished results). Thus, the transport of sodium and water is unaffected and the changes in absorption follow rather than precede the defect in mineralization. The decrease in calcium absorption may therefore be brought about by an unidentified mechanism which links the efficiency of absorption to the need of the skeleton for calcium (Nicolaysen, 1943; Morrissey & Wasserman, 1971). If this interpretation is correct, there will be a tendency for the animals to become hypercalcemic when bone mineral accretion is impaired. Such a hypercalcaemia is observed.

**Calcium balance**

Despite large changes in bone resorption, the net calcium balance changed only little under Cl$_2$MDP and the lower doses of EHDP. The animals therefore seem unable to increase the net uptake of calcium by bone to an appreciable degree and instead respond to a decrease in bone resorption rate by a corresponding decrease in bone formation rate. The correlation observed between bone formation ($V_{0+}$) and resorption ($V_{0-}$) is so close that it is unlikely to
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occur because of separate, but equal and direct effects of the diphosphonate on each process. It seems more likely that some mechanism exists for coupling the two processes in the body (Harris & Heaney, 1969).

Comparison of the two diphosphonates

The effects of EHDP and Cl₂MDP differ in several respects. At an equal dose Cl₂MDP decreases bone resorption more than EHDP. This confirms studies in tissue culture (Russell et al., 1970; J. J. Reynolds, C. Minkin, D. B. Morgan, D. Spycher & H. Fleisch, unpublished results), on PTH-induced increases in plasma calcium (Russell et al., 1970) and on prevention of immobilization osteoporosis (Mühlbauer et al., 1971). At low doses the effect of the two compounds on calcium balance is similar despite the fact that Cl₂MDP decreases bone turnover to a larger extent than EHDP; at the high doses, however, EHDP causes a marked decrease of $V_{o+}$ due to an inhibition of bone and cartilage mineralization, whereas Cl₂MDP does not. These differences may be of great importance when the administration of the two diphosphonates to humans is considered.

Mechanism of action of diphosphonates

At present the only identified properties of diphosphonates which can explain their effects on calcium metabolism are those on the growth and dissolution of apatite crystals in vitro. However, it is difficult to account for all the results in simple physicochemical terms. Thus, the greater effect of Cl₂MDP than EHDP on bone resorption contrasts with its weaker effect on crystal dissolution in vitro (Russell et al., 1970). The difference between the two diphosphonates on bone mineralization cannot easily be explained since they do not have greatly different effects in vitro (Fleisch et al., 1970). It is possible that the differences between the two compounds are due to a difference in distribution within bone, or that other effects exist which have not yet been identified.

Administration of diphosphonates to humans

The observation that diphosphonates diminish bone turnover in the rat has led to preliminary investigations of the use of these compounds in human conditions where bone resorption is increased, such as Paget’s disease. Recent results show that EHDP given to patients with Paget’s disease causes biochemical changes consistent with a decrease in the bone turnover (Smith, Bishop & Russell, 1971). In one patient with myositis ossificans, EHDP also caused a decrease in bone destruction rate and bone formation rate as measured by radioactive calcium kinetics (Weiss, Fisher & Phang, 1971). The doses used were between 2.5–5 mg of P of EHDP kg⁻¹ day⁻¹ given orally which, since the absorption of EHDP in man is in the order of 3–5% (W. R. Michael & W. R. King, unpublished results), would represent a systemic dose of about 0.1–0.2 mg P/kg. Our finding that Cl₂MDP is, for equal doses, more effective than EHDP in decreasing bone resorption in animals but that it impairs the mineralization of bone to a lesser extent when given in large amounts, suggests that Cl₂MDP might be preferable in diseases with an increased resorption of bone.

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REFERENCES


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