Influence of a Diphosphonate on Serum Calcium Homeostasis[1] (37331)

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Introduction. Previous studies have shown that compounds with P–C–P bonds, called diphosphonates, can inhibit both the formation (1–3) and dissolution (1, 4, 5) of apatite crystals *in vitro*. It appears that they have similar properties also *in vivo*. Thus, in rats, they inhibit soft tissue calcification induced by vitamin D administraton (1–3) and other means (6), and reduce the formaton of urinary stones (7). When given in large amounts, certain diphosphonates induce rickets (8–11). Furthermore, diphosphonates prevent the endogenous bone resorption as well as the resorption induced by parathyroid hormone in tissue culture (1, 4, 5), prevent the resorption induced by a low calcium diet in growing rats as measured by using kinetic analysis (10), and prevent the hypercalcaemia induced by PTH in parathyroidectomised rats (4, 5). Finally, they prevent the osteoporosis induced by nerve section in rats (12–14).

Such effects raise the question of the influence of diphosphonate treatment on extracellular calcium homeostasis. This is of importance because of the possible therapeutic use of diphosphonates in certain clinical conditions. Indeed, ethane-1-hydroxy-1,1-diphosphonate (EHDP) has already been shown to inhibit or completely block the progression of ectopic calcification in myositis ossificans (15) and to reduce bone turnover as assessed by urinary and plasma hydroxyproline and plasma alkaline phosphatase in Paget's disease (16).

The aim of the present study was to examine the variation of plasma calcium ($[Ca]_p$) and phosphate ($[P]_p$) in response to acute addition or removal of calcium to or from the extracellular pool in rats treated with one of the diphosphonates, dichloromethylene diphosphonate (Cl_2MDP).

Materials and Methods. Experiments were carried out on female Wistar rats weighing 150–200 g, fed with a commercial chow (No. 194, Nafag, Gossau) containing 1.3 g Ca and 1.0 g P per 100 g dry weight (as confirmed by us on samples dried at 100° for 48 hr). The animals of the experimental groups received disodium dichloromethylene diphosphonate (Cl_2MDP) for 3 days at a daily dose of 10 mg P/kg body wt. The substance was dissolved in 0.15 *M* NaCl and injected sc in a volume of 2.0 ml/kg body wt between 10 and 11 o'clock AM. Nontreated animals received 2.0 ml/kg body wt of 0.15 *M* NaCl sc. On the third day of treatment, all animals were fasted from 4.0 o'clock PM but had free access to distilled water. On the fourth day, a first blood sample (0.2 ml) was taken between 8 and 9 AM from the vein of the foot of a hind limb, the rat being conscious in a restrictive cage. After this first blood sampling, nontreated and Cl_2MDP-treated rats underwent the following treatments:

Experiment I. 20 mg Ca/kg body wt was given ip as an 0.1 *M* $CaCl_2$ solution adjusted to pH 7.4 with 1 *N* NaOH. The volume injected was 5.0 ml/kg body wt. Blood was taken 15 min and 6 hr after the injection of calcium.

Experiment II. 20 mg Ca/kg body wt was given by iv infusion within 60 min. The solution contained $CaCl_2$ (0.025 *M*) and NaCl (0.10 *M*). It was delivered at a rate varying between 3.8 and 4.0 ml/hr through a tail vein. During the time of the infusion, the animals were also kept in the restrictive cages. Blood was taken at the end of the infusion and 5 hr later.

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Experiment III. 100 mg P/kg was given ip as an 0.332 *M* Na_2HPO_4 solution adjusted to pH 7.4 with 1 N HCl. The volume injected corresponded to 10 ml/kg body wt. Blood was taken 1 and 6 hr after the phosphate injection.

Chemical determination. Plasma calcium ($[Ca]_p$) was determined by atomic absorption spectroscopy (Perkin Elmer Model 290 B) after dilution of the samples with 0.5% $LaCl_3$; phosphorus in plasma ($[P]_p$) and urine was determined colorimetrically as phosphomolybdate after reduction with a 10% ascorbic acid solution (17).

Statistical evaluation. All experimental results are expressed as mean ± standard error of the mean (SEM). Significance of difference between groups was determined by the Students *t* test. Significance of the change in $[Ca]_p$ and $[P]_p$ within one group was evaluated by testing the significance of the means of the differences compared to zero.

Results. The data in Table I show the variation in $[Ca]_p$ and $[P]_p$ in response to the ip injection of 20 mg Ca/kg. The preinjection $[Ca]_p$ for the Cl_2MDP-treated group is significantly lower than for the untreated group. Fifteen minutes after the Ca injection, the absolute increase in $[Ca]_p$ was greater in Cl_2MDP-treated rats (+3.48 ± 0.17 vs 2.40 ± 0.39 in nontreated animals). Six hours after the Ca injection, the plasma calcium of the Cl_2MDP-treated rats was again significantly lower than in the untreated group. The plasma phosphate of Cl_2MDP-treated animals was slightly lower ($p < 0.05$) than in the untreated animals before the calcium challenge and significantly lower ($p < 0.001$) 6 hr after the calcium injection.

The data presented in Table II show the effect of Cl_2MDP treatment on the variation in $[Ca]_p$ and $[P]_p$ in response to the same amount of calcium given by continuous iv infusion over 1 hr. In this experiment, no significant initial difference could be detected between the two groups. At the end of the Ca infusion, only the Cl_2MDP-treated group presented a significant ($p < 0.001$) rise in $[Ca]_p$. As in the previous experiment, the Cl_2MDP-treated rats had significantly ($p < 0.01$) lower $[Ca]_p$ 6 hr after the beginning of the calcium infusion and, 5 hr after the

TABLE I. Effect of Cl_2MDP Treatment on the Change in Plasma Calcium $[Ca]_p$ and Phosphate $[P]_p$ in Response to an Intraperitoneal Calcium Injection (20 mg/kg).

Blood sample no.:	1	2	Δ 2—1	3	Δ 3—1
Time:	15 min pre-injection	15 min post-injection		6 hr post-injection	
$[Ca]_p$ (mg %)[c]					
nontreated	10.80	13.20	+2.40, $p^b<0.01$	9.72	−1.08, $p^b<0.05$
($n = 5$)[d]	±0.22	±0.20	±0.39	±0.21	±0.35
Cl_2MDP-treated	10.10	13.58	+3.48, $p^b<0.001$	9.18	−0.92, $p^b<0.01$
($n = 5$)	±0.20	±0.25	±0.17	±0.09	±0.15
	$p^a<0.02$	*	$p^a<0.05$	$p^a<0.05$	*
$[P]_p$ (mg %)[c]					
nontreated	8.30	8.48	+0.18, $p^b>0.05$	8.46	+0.16, $p>0.05$
($n = 5$)	±0.19	±0.58	±0.59	±0.37	±0.40
Cl_2MDP-treated	7.14	6.68	−0.46, $p^b>0.05$	6.16	−0.98, $p>0.05$
($n = 5$)	±0.39	±0.41	±0.58	±0.09	±0.41
	$p^a<0.05$	$p^a<0.05$	*	$p^a<0.001$	*

[a] Significance of difference between Cl_2MDP-treated and nontreated group.

[b] Significance of the difference between sample No. 2 (or 3) and 1 estimated by paired sample analysis.

[c] Mean ± standard error of the mean (SEM).

[d] n = Number of animals.

* $p^a>0.05$.

TABLE II. Effect of Cl_2MDP Treatment on the Change in Plasma $[Ca]_p$ and Phosphate $[P]_p$ in Response to an Intravenous Calcium Infusion (20 mg/kg over 1 hr).

Blood sample no.:	1	2	Δ 2—1	3	Δ 3—1
Time:	15 min pre-infusion	End infusion		5 hr after end infusion	
$[Ca]_p$ (mg %)[c]					
nontreated	10.34	10.52	+0.18, $p^b>0.05$	9.46	−0.88, $p^b<0.001$
($n=12$)[d]	±0.12	±0.20	±0.18	±0.17	±0.18
Cl_2MDP-treated	9.97	11.14	+1.17, $p^b<0.001$	8.64	−1.33, $p^b<0.001$
($n=12$)	±0.15	±0.25	±0.20	±0.17	±0.14
	*	*	$p^a<0.01$	$p^a<0.01$	*
$[P]_p$ (mg %)[c]					
nontreated	8.34	7.59	−0.75, $p^b<0.05$	7.32	−1.02, $p^b<0.05$
($n=12$)	±0.25	±0.20	±0.31	±0.28	±0.40
Cl_2MDP-treated	7.87	7.55	−0.41, $p^b>0.05$	6.49	−1.47, $p^b<0.001$
($n=11$)	±0.27	±0.24	±0.30	±0.23	±0.25
	*	*	*	$p^a<0.05$	*

[a] Significance of difference between Cl_2MDP-treated and nontreated group.

[b] Significance of the difference between sample No. 2 (or 3) and 1 estimated by paired sample analysis.

[c] Mean ± standard error of the mean (SEM).

[d] n = Number of animals.

* $p^a>0.05$.

end of the infusion, was significantly lower in the Cl_2MDP-treated group than in the controls.

In Table III, the data concerning the phosphate injection are presented. As in the first experiment, the Cl_2MDP-treated rats had a lower initial $[Ca]_p$ than untreated animals. This difference in $[Ca]_p$ was still present 6 hrs after the injection of phosphate. The increase in $[P]_p$ was smaller after 1 hr in the Cl_2MDP-treated animals.

Discussion. These results confirm previous observations (10) that rats treated with Cl_2MDP tend to have a lower $[Ca]_p$ as well as a lower $[P]_p$. These changes could be explained by the known action of diphosphonates to decrease bone resorption, presumably because of a primary effect on crystal dissolution. The diminished bone resorption would lead to a secondary rise in PTH, which would limit the hypocalcaemia due to its effect on renal tubular reabsorption of calcium and would lead to the observed hypophosphatemia.

It is probable that rapid changes in blood calcium, as indicated in this paper, are counteracted by changes in net uptake of calcium by bone induced by changes in PTH and calcitonin secretion. These compensatory responses of net calcium uptake by bone are, at least partly, due to changes in bone resorption. Since the latter is impeded by Cl_2MDP, it is not surprising that the animals cannot cope as efficiently with either a load (Tables I and II), or a withdrawal (Table III) of calcium as did the untreated controls. The fact that plasma phosphate increases less after a phosphate load in the Cl_2MDP-treated animals (see Table III) could also be explained by the same basic mechanism, since a secondary increase of parathormone in these animals could lead to a decrease in blood phosphate.

It should be emphasized that the doses of Cl_2MDP used exceed those used clinically (15, 16). It would, however, be worthwhile investigating whether similar impairment of calcium homeostasis occurs in humans treated with Cl_2MDP.

Summary. Disodium dichloromethylene diphosphonate (Cl_2MDP) administered at 10 mg P/kg/day sc for 3 days to rats leads to a

TABLE III. Effect of Cl_2MDP Treatment on the Change in Plasma Calcium $[Ca]_p$ and Phosphate $[P]_p$ in Response to a Phosphate Injection (100 mg/kg P ip).

Blood sample no.:	1	2	Δ 2—1	3	Δ 3—1
Time:	15 min pre-injection	1 hr post-injection		6 hr post-injection	
$[Ca]_p$ (mg %)[c]					
nontreated	10.79	8.78	—2.01, $p^b<0.001$	9.70	—1.09, $p^b<0.001$
($n = 22$)[d]	±0.16	±0.15	±0.24	±0.09	±0.14
Cl_2MDP-treated	10.20	7.37	—2.83, $p^b<0.001$	8.29	—1.91, $p^b<0.001$
($n = 10$)	±0.18	±0.27	±0.27	±0.30	±0.32
	$p^a<0.05$	$p^a<0.001$	*	$p^a<0.001$	$p^a<0.01$
$[P]_p$ (mg %)[c]					
nontreated	7.11	11.51	+4.40, $p^b<0.001$	6.43	—0.68, $p^b<0.001$
($n = 22$)	±0.14	±0.19	±0.22	±0.12	±0.12
Cl_2MDP-treated	6.71	10.23	+3.52, $p^b<0.001$	5.80	—0.91, $p^b<0.02$
($n = 10$)	±0.27	±0.33	±0.40	±0.26	±0.32
	*	$p^a<0.001$	$p^a<0.05$	$p^a<0.02$	*

[a] Significance of difference between Cl_2MDP-treated and nontreated group.

[b] Significance of the difference between sample No. 2 (or 3) and 1 estimated by paired sample analysis.

[c] Mean ± standard error of the mean (SEM).

[d] n = Number of animals.

* $p^a>0.05$.

decreased blood calcium and decreased blood phosphate. The treated animals responded to a calcium load administered ip or iv with a larger increase in blood calcium and to a phosphate load with a larger decrease in blood calcium. It is suggested that this diminished efficiency in regulating blood calcium is due to the inhibiting effect of the diphosphonate on bone resorption.

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