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The Effect of Two Diphosphonates on the Resorption of Mouse Calvaria *in vitro*

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Two diphosphonates, disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) and disodium dichloromethylene diphosphonate (Cl_2MDP), inhibit cell-mediated bone resorption of mouse calvaria cultivated for 48 hours *in vitro*, when the compounds are added to the medium. Cl_2MDP is more effective than EHDP over the dose range 0–16 μg P/ml. Pyrophosphate and imidodiphosphate do not block bone resorption at comparable dose levels. When the two diphosphonates are injected into mice *in vivo* before explants are prepared, subsequent bone resorption *in vitro* is considerably reduced; at a dose level of 10 μg P/g body weight of Cl_2MDP it is almost completely blocked. This effect is rapid and persists for several days. The implications of these results and the method of testing inhibitors of bone resorption by the combined *in vivo/in vitro* method are discussed.

Key words: Diphosphonates — Bone resorption — Mouse — Pyrophosphate — Tissue culture — ^{45}Calcium.

Deux diphosphonates, le disodium-éthane-1-hydroxyle-1,1-diphosphonate (EHDP) et le disodium dichlorométhylène diphosphonate (Cl_2MDP), inhibent la résorption osseuse, induite par des cellules au niveau de calottes craniennes, cultivées pendant 48 heures *in vitro*, lorsque ces substances sont ajoutées au milieu. Le Cl_2MDP est plus actif que l'EHDP, à des doses variant 0—16 μg P/ml. Le pyrophosphate et l'imidodiphosphate n'inhibent pas la résorption osseuse à des doses comparables. Lorsque les deux diphosphonates sont injectés à des souris *in vivo* avant mise en culture, la résorption osseuse observée *in vitro* est considérablement réduite: à une dose de 10 μg P/g de poids corporel de Cl_2MDP, elle est presque totalement inhibée. Cet effet est rapide et dure plusieurs jours. Les conséquences de ces résultats et la méthode d'essai d'inhibiteurs de la résorption osseuse par la méthode combinée *in vivo/in vitro* sont envisagées.

Zwei Diphosphonate, Dinatrium-äthan-1-hydroxy-1,1-diphosphonat (EHDP) und Dinatrium-Dichloromethylendiphosphonat (Cl_2MDP), hemmen zellbedingte Knochenresorption von Mäuseschädeldächern, welche während 48 Std *in vitro* kultiviert worden waren, wenn diese Substanzen dem Nährmedium zugegeben werden. Im Dosierungsbereich von 0—16 μg P/ml ist Cl_2MDP wirksamer als EHDP. Pyrophosphat und Imidodiphosphat blockieren die Knochenresorption bei entsprechenden Dosen nicht. Wenn die zwei Diphosphonate Mäusen *in vivo* injiziert werden, bevor das Explantat hergestellt wird, ist die nachfolgende Knochenresorption *in vitro* stark vermindert; bei einer Dosierung von 10 μg P/g Körpergewicht von Cl_2MDP ist die Resorption fast gänzlich blockiert. Diese Wirkung erfolgt rasch und dauert während einigen Tagen an. Die Folgerungen aus diesen Ergebnissen sowie das Verfahren, Knochenresorptionshemmer mittels kombinierter *in vivo/in vitro*-Methode zu prüfen, werden diskutiert.

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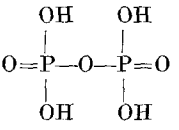
Introduction

Compounds which diminish bone resorption are of great interest because of their clinical application in conditions where bone resorption is increased. Recently, attention has been brought to a new class of compounds, the diphosphonates. Work on pyrophosphate (PP_i) has shown that this compound decreases the dissolution of hydroxyapatite *in vitro* (Fleisch *et al.*, 1966a, b); more recently it was found that the diphosphonates, which are related in structure to PP_i but with a P—C—P bond in place of a P—O—P bond, have a similar action (Fleisch *et al.*, 1968, 1969a; Russell *et al.*, 1970). In living systems diphosphonates decrease bone resorption in several cases where PP_i is ineffective: thus they inhibit resorption induced by parathyroid hormone (PTH) in tissue culture (Fleisch *et al.*, 1968, 1969a; Russell *et al.*, 1970), they prevent the increase of blood calcium induced by PTH in rats (Fleisch *et al.*, 1968, 1969a; Russell *et al.*, 1970) and they diminish bone turnover in the intact rat (Gasser *et al.*, 1972). The difference in the effectiveness of PP_i and diphosphonates *in vivo* might be that PP_i is easily hydrolysed in the body whereas the diphosphonates are completely resistant to destruction.

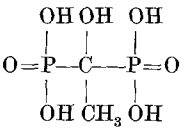
In the present study we have compared the effects of three classes of compounds with a P—X—P bond, on the rate of nonstimulated bone resorption of mouse calvaria *in vitro*. The compounds studied were pyrophosphate (with a P—O—P bond), imidodiphosphate (with a P—N—P bond) which has been shown also to inhibit dissolution of hydroxyapatite (Robertson and Fleisch, 1970), and two diphosphonates (with a P—C—P bond) namely, disodium dichloromethylene diphosphonate (Cl_2MDP) and disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP). Fig. 1 shows the formulae of these compounds.

In the first series of experiments we compared the effect of the compounds on bone resorption when they were added to the culture medium (*in vitro* experiments). In a further series of experiments we compared the effect of EHDP

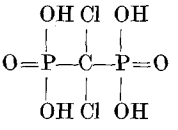
Abbreviations: Pyrophosphate, PP_i; imidodiphosphate, IDP; disodium dichloromethylene diphosphonate, Cl_2MDP; disodium ethane-1-hydroxy-1,1-diphosphonate, EHDP.



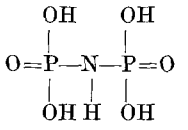
Inorganic pyrophosphoric acid (PP_i)



Ethane-1-hydroxy-1,1-diphosphonic acid (EHDP)



Dichloromethylene diphosphonic acid (Cl_2MDP)



Imidophosphoric acid (IDP)

Fig. 1. Structural formulae

and Cl_2MDP when the compounds were injected into the animals *in vivo* and the resorption was measured subsequently during *in vitro* culture (*in vivo/in vitro* experiments). By varying the time between the injection of the diphosphonate and the explantation we were able to investigate how quickly and for how long the compounds acted on bone *in vivo*.

Abstracts of part of these studies have appeared (Reynolds and Morgan, 1970; Reynolds, 1971; Fleisch *et al.*, in press).

Methods and Materials

The general method of bone organ culture that was used in these studies has been described in detail elsewhere (Reynolds and Dingle, 1970); only minor changes were made where necessary.

NMRI mice from an inbred colony were injected subcutaneously with $^{45}Ca\ Cl_2$ (specific activity 11 mCi/mg Ca) on the day of birth (usually 1.0 μCi per mouse, unless otherwise stated). The mice were killed, unless otherwise stated, four days after the pulse of isotope. The calvaria (frontal and parietal bones) were removed aseptically and paired half-calvaria were cultivated for 48 h as described previously. The volume of medium was 5.5 ml, so that the medium pool of calcium acted as a trap for essentially all of the isotope released from an explant during the culture period (as discussed in Reynolds and Minkin, 1970). Resorption was assessed by measuring the liberation of ^{45}Ca into the medium. At the end of the experiments each half bone was dissolved in 1 ml of formic acid. The ^{45}Ca contents of the media and bone solutions were measured in an automatic liquid scintillation counter, Packard Model 3950.

The ^{45}Ca released from an explant is liberated by two mechanisms, an exchange of the isotope with cold calcium in the medium and net dissolution of mineral due to cell-mediated resorption (Reynolds and Minkin, 1970). The latter was assessed by subtracting the amount of isotope released into the culture medium by a dead explant from the amount released by its living paired half-calvaria. Dead explants were prepared by the procedure of freezing and thawing three times. The justification for this procedure has been discussed elsewhere (Reynolds, 1971; Reynolds, 1972; Reynolds and Minkin, 1970), and a similar procedure for calculating resorption has also been used by other workers (for example, see Raisz and Niemann, 1969).

Inorganic pyrophosphate was obtained from Merck, Darmstadt, Germany, and imidodiphosphate was a gift from Professor R. G. Yount, University of Pennsylvania, Philadelphia. Cl_2MDP and EHDP were supplied by the Procter and Gamble Company, Cincinnati, USA. For the *in vitro* experiments test substances were dissolved into the culture medium before sterilisation by Millipore filtration. For the experiments in which the substances were injected into mice, the vehicle used was Tyrode's solution, which was also used for the control injection. The doses of each compound are expressed in terms of phosphorus content.

Unless otherwise stated all results are expressed as means ± the standard error of the mean (S.E.M.). Statistical significance was assessed by means of Student's *t* test.

Results

The Assessment of ^{45}Ca Release and of the Effects of the Compounds on It

Fig. 2a shows that the release of ^{45}Ca from live and dead half-calvaria of untreated mice is directly proportional to the ^{45}Ca content of the bone at the time of explantation. The latter figure is calculated as the sum of the ^{45}Ca in the medium and in the bone at the end of the experiment. The variation in bone isotope content is probably partly the result of unequal injections of isotope; the small size and fragility of the new-born mice make injections difficult. Fig. 2b shows that the variation in the ^{45}Ca content of the medium can be re-

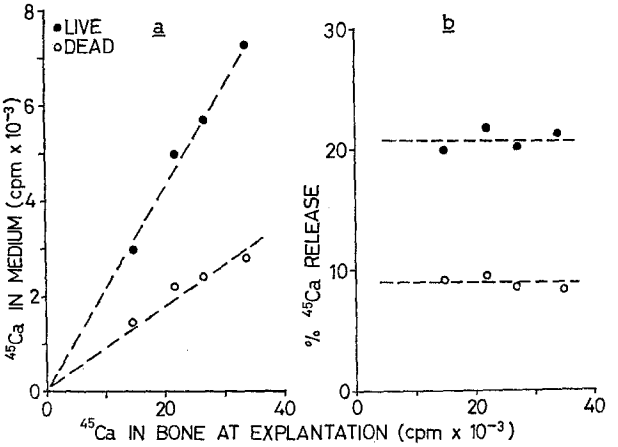


Fig. 2a and b. The relation between the ^{45}Ca content of the bones at explantation and the subsequent release from live and dead bones. The release of ^{45}Ca is expressed in (a) as the total ^{45}Ca content of the medium and (b) the ^{45}Ca content of the medium as a percentage of the ^{45}Ca content of the bone at explantation. The results have been grouped according to the ^{45}Ca content of the bone, and the values shown are the mean values in each group. The dotted values in (b) are the average values for the % release of ^{45}Ca, which was 20.8% for the 57 live bones and 9.1% for the 54 dead bones

moved by calculating the ^{45}Ca release as a percentage of the ^{45}Ca content of the bone at the time of explantation (% ^{45}Ca released).

The effect of the various compounds on % ^{45}Ca release is based on a comparison of treated (T) and control (C) half-calvaria or of bones from treated (T) or control (C) animals.

Effect of Pyrophosphate, a Diphosphonate and Imidodiphosphate Added to the Medium, on the Release of ^{45}Ca from Living Half-Calvaria in vitro

Fig. 3 shows the effect of PP_i, IDP and Cl_2MDP added to the culture medium on the release of ^{45}Ca from living explants. All compounds were tested at a dose of 4 μg P/ml. Only Cl_2MDP significantly reduced the release of isotope. Imidodiphosphate increased the release of ^{45}Ca.

Effect of Different Doses of Cl_2MDP and EHDP Added to the Medium on the Release of ^{45}Ca from Live and Dead Half-Calvaria in vitro

The study was performed with a block design: on any one day, the half-calvaria were explanted from twelve four-day-old prelabelled mice from one litter. One of each pair of half-calvaria was treated with one of the twelve doses of a compound added to the medium, the other half was used as the untreated control. The two diphosphonates were tested on alternative days and the study was continued until each dose of each compound had been tested five times. The effects of these twelve different doses of either Cl_2MDP or EHDP added to the culture medium of live bones are illustrated in Fig. 4. For both compounds there was a dose-dependent inhibition of the release of ^{45}Ca, and Cl_2MDP was

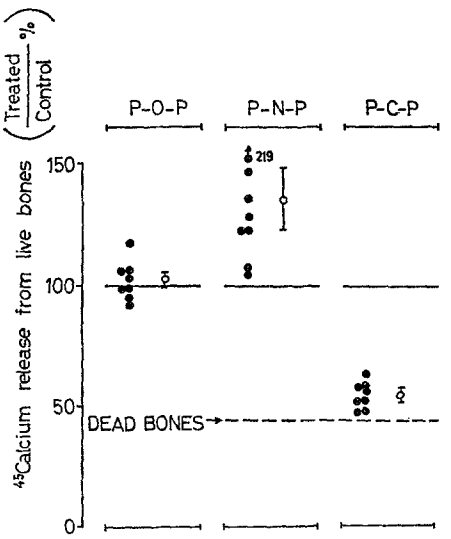


Fig. 3. The effect of pyrophosphate (P—O—P), imididophosphate (P—N—P) and dichloromethylene diphosphonate (P—C—P) on the release of ^{45}Ca from live bones *in vitro*. The compounds were added as the sodium salt to the medium of one of each pair of half-calvaria at a concentration of 4 μg P/ml, the paired half-calvaria serving as controls. The individual results and the mean ± 1 SEM are shown. For comparison the level of release from dead bones is shown as a dotted line (data from Fig. 2b)

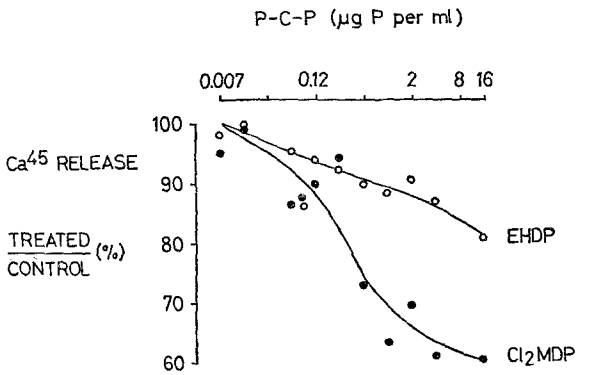


Fig. 4. The effect of twelve concentrations of Cl_2MDP and EHDP on the release of ^{45}Ca from live half-calvaria into the medium during 48 h cultivation. The paired half-calvaria served as controls. Each point on the curves is the mean of 5 results; the standard error of the mean was of the order of 6%. The curves are arbitrary sketches

more effective than EHDP. The dose response curves are complex and do not appear to be parallel, which may indicate differences in the mode of action of the two compounds.

However, before attributing the difference in potency of the two diphosphonates to a differential effect of the compounds on cell-mediated resorption, it was necessary to show that these compounds had no effect on the release

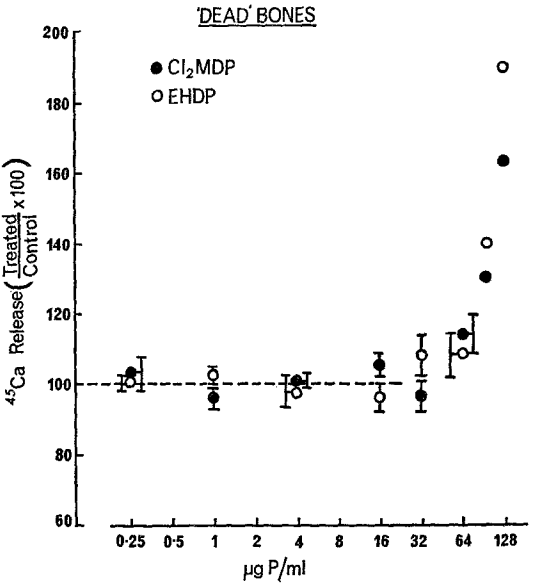


Fig. 5. The effect of Cl_2MDP and EHDP on the release of ^{45}Ca from dead half-calvaria into the medium during 48 h cultivation. Paired dead half-calvaria served as the controls. Each point on the curve represents the mean of either 3 or of 4 to 6 results; in the latter case the mean $\pm$ 1 SEM is also indicated

of ^{45}Ca from dead calvaria. Large doses only of the compounds (32 μg P per ml or more) increased the release of ^{45}Ca from the dead bones (Fig. 5) and the effects of these large doses of the compounds on live bones have therefore been excluded from consideration in Fig. 4. From the data obtained with the live and dead control halves in the experiments illustrated in Figs. 4 and 5 we calculated that dead bones release on the average 44% of the amount of isotope released by the live bones. Since live bones treated with 16 μg P/ml of Cl_2MDP release 57% of the ^{45}Ca released by untreated live bones, it could be calculated that this dose of Cl_2MDP blocked 76% of cell-mediated resorption.

Effect of Cl_2MDP and EHDP on Bone in vivo *as Observed* in vitro

The aim of these experiments was to test the relative effectiveness of Cl_2MDP and EHDP in blocking bone resorption in explants *in vitro* when the mice were previously treated *in vivo* with these compounds. Since the ^{45}Ca was always injected within 24 h of birth and the administration of the compound always started four days later the explantation had to be performed in mice of various ages. The various characteristics of bones of the two extreme ages of 4 and 7 days are presented in Table 1. The amount of isotope in the explants is the same at the two ages and is not labile during the experimental period, and the amount of cell-mediated resorption *in vitro* is not much less in the explants from 7-day-old mice than in the explants from 4-day-old mice.

Table 1. The release of ^{45}Ca from half-calvaria taken from mice aged either 4 or 7 days and cultivated *in vitro* for 48 h. All mice received a subcutaneous injection of 12.5 μCi ^{45}Ca on the day of birth. The results are given as means ± SEM

	Explants from mice 4 days old	Explants from mice 7 days old
Number of mice	6	6
Weight at age 4 days (g)	2.5 ± 0.1	2.5 ± 0.1
Weight at age 7 days (g)	—	4.8 ± 0.1
^{45}Ca released by living half-calvaria (%)[a]	14.7 ± 0.6	13.6 ± 0.9
^{45}Ca released by paired dead explants (%)[a]	4.6 ± 0.3	5.3 ± 0.2
Cell-mediated resorption (%)[b]	10.1 ± 0.6	8.3 ± 0.9
Total isotope per calvarium (c.p.m.)	807000 ± 38000	794000 ± 19000

[a] Expressed as percentage total bone isotope.
[b] Calculated as the difference, % ^{45}Ca release (live half—dead half), for each pair of bones.

Table 2. Resorption *in vitro* of half-calvaria from mice aged 7 days injected subcutaneously either with Cl_2MDP (10 μg P/g body weight) or an equal volume of vehicle fluid when aged 4, 5 and 6 days. All mice received a subcutaneous injection of 1.0 μCi ^{45}Ca on the day of birth. The *in vitro* culture period was 48 h. The results are given as means ± SEM

	Controls	Treated with Cl_2MDP
Number of mice	6	6
Weight at age 4 days (g)	3.6 ± 0.1	3.6 ± 0.1
Weight at age 7 days (g)	4.9 ± 0.2	5.0 ± 0.1
Dose of Cl_2MDP (μg P/g/day)	0	10.0
^{45}Ca released by living half-calvaria (%)[a]	13.6 ± 0.9	4.6 ± 0.2
^{45}Ca released by paired dead explants (%)[a]	5.4 ± 0.4	3.3 ± 0.2
Cell-mediated resorption (%)[b]	8.2 ± 0.7	1.3 ± 0.1
Total isotope per calvarium (c.p.m.)	64700 ± 3900	61300 ± 3200

[a] Expressed as percentage total bone isotope.
[b] Calculated as the difference, % ^{45}Ca release (live half—dead half), for each pair of bones.

Table 2 shows the data from an experiment in which a litter of twelve mice received either 10 μg P/g of body weight of Cl_2MDP or an equal volume of control vehicle daily, during a three day period from age 4 to 7 days. The amount of

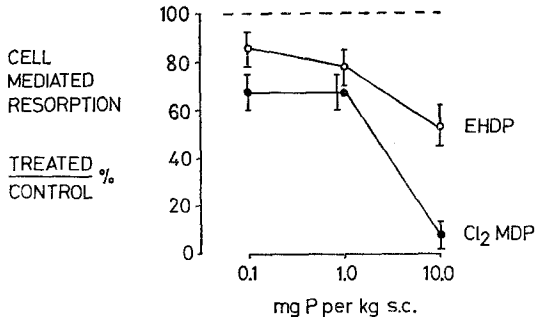


Fig. 6. The effect of three doses of Cl_2MDP and EHDP on the cell-mediated resorption of half-calvaria cultivated *in vitro* for 48 h. The compounds were given as a single subcutaneous injection when the mice were 4 days old and the half-calvaria were explanted 24 h later. At each dose the effect is expressed as the ratio of the cell-mediated resorption in the bones of the treated animals to the mean value for the bones of control animals studied at the same time. Each value shown is the mean ± 1 SEM of 6 results

cell-mediated resorption in the explants taken from treated mice was only about 15% of that of the control group of mice. The ^{45}Ca release was also less from the dead bones of treated animals than of control animals, as might be expected since bone turnover has been reduced. The total amount of isotope in the calvaria at explantation was not different in the animals which had received Cl_2MDP.

Fig. 6 shows the effect of various doses of Cl_2MDP and EHDP, when given to the mice *in vivo*, on the subsequent cell-mediated resorption *in vitro*. A single dose of either 0.1, 1 or 10 μg P/g of body weight of diphosphonate was given to the mice on the fourth day after the isotope pulse. The calvaria were explanted 24 h later and cultivated for 48 h. Each calvarium was divided into two halves and one of each pair of halves was frozen and thawed three times. Cell-mediated bone resorption for each animal was again calculated as the difference of the ^{45}Ca release between the two halves. In any one experiment, the effect of the same dose of each of the compounds was compared with the effect of the control injection and all the values have been plotted as a percentage of the average value for the bones from the control animals in that experiment. Cl_2MDP was again more effective than EHDP; at the dose of 10.0 μg P/g body weight, Cl_2MDP had blocked nearly all cell-mediated resorption.

Fig. 7 illustrates the relative speed of onset and the duration of the inhibition of resorption by Cl_2MDP and EHDP, when given to the mice *in vivo*. A single injection of either Cl_2MDP or EHDP (10 μg P/g body weight) was given s.c. to the mice at four days of age. The calvaria were explanted 2 h, 1, 2 and 3 days later, and cultivated for 48 h. The results are expressed as in the previous experiment. Even at the earliest time interval of 2 h both diphosphonates had largely blocked cell-mediated bone resorption. The effect persisted for 3 days, which was the longest time investigated. Again, Cl_2MDP was more effective than EHDP, although after three days the difference was nearly abolished.

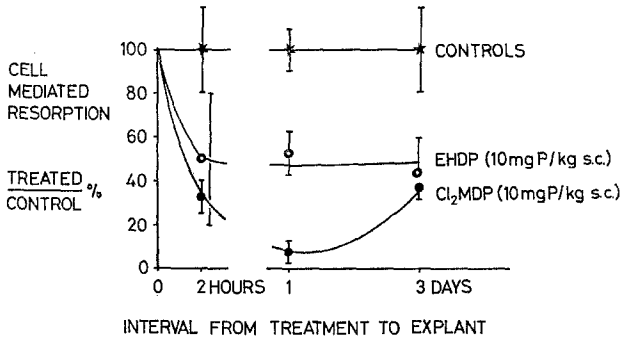


Fig. 7. The effect of Cl_2MDP and EHDP on cell-mediated resorption of half-calvaria *in vitro* in relation to the interval of time between the injection of the compounds and explantation. The compounds were given as a single subcutaneous injection (10 μg P/g body weight) when the mice were 4 days old. The calvaria were explanted 2 h, 1 day or 3 days later and cultured for 48 h. The results are expressed as in Fig. 6. Each value is the mean ± 1 SEM for 6 results

Discussion

The results presented in this paper show that two diphosphonates, EHDP and Cl_2MDP, both inhibit the non-stimulated (endogenous) bone resorption of mouse calvaria *in vitro*, either when the compounds are added to the culture medium or when they are administered to the animals *in vivo* before explantation. These two compounds have previously been shown to inhibit the effect of parathyroid hormone to stimulate bone resorption *in vitro* (Fleisch *et al.*, 1968, 1969a; Russell *et al.*, 1970). We conclude that they act directly on bone and do not merely interfere with the action of parathyroid hormone.

When added to the medium both EHDP and Cl_2MDP caused a progressive inhibition of cell-mediated bone resorption over the dose range of 0–16 μg P/ml. Cl_2MDP was much more potent than EHDP at all dose levels. The dose response curves for Cl_2MDP and EHDP are not parallel, but their form (Fig. 4) precludes any conclusive statements about whether they act by the same or different mechanisms. At doses greater than 32 μg P/ml, both diphosphonates increase the release of isotope from dead explants. The explanation could be that at high doses of diphosphonates sufficient calcium is complexed to considerably alter the exit of calcium from the bone to the medium. On the other hand, it is possible that the diphosphonates liberate colloidal calcium phosphate from the calvaria into the medium (peptisation) since diphosphonates have such an effect on hydroxyapatite crystals *in vitro* (Robertson *et al.*, in preparation).

As found previously (Fleisch *et al.*, 1969a; Russell *et al.*,1970), pyrophosphate does not inhibit resorption (Fig. 3) when it is added to the medium used for bone culture. It was interesting to find that imidodiphosphate was also ineffective in blocking endogenous bone resorption even though it has an inhibitory effect on the dissolution of calcium phosphate *in vitro* (Robertson and Fleisch, 1970). The reason for the increased release of isotope from explants treated with imidodiphosphate is not known. Perhaps this effect is comparable with the effect of high doses of diphosphonates on dead bone that is discussed above. It is possible that the difference in effect between diphosphonates, pyrophosphate

and imidodiphosphate is due to the hydrolysis of the latter two before they can reach the site of action. Diphosphonates, on the other hand, are known to be completely resistant to enzymatic hydrolysis.

In order to examine whether, and if so, how quickly and for how long, the two diphosphonates inhibit bone resorption, these compounds were injected into the animals four days after they had been given the pulse of ^{45}Ca. The ability of explants from such animals to resorb *in vitro* was compared with explants from untreated animals. This type of approach has been used successfully before but with other criteria than the release of ^{45}Ca. Thus, the *in vitro* metabolism of bone from normal animals has been compared with that of bone from animals that had been treated with either parathyroid hormone (Borle *et al.*, 1960; Flanagan and Nichols, 1964; Johnston *et al.*, 1965) or vitamin D (Au and Bartter, 1966), or from animals that had been parathyroidectomised (Cooper *et al.*, 1965). That the technique described in this paper is suitable to test the effects of agents on bone resorption *in vivo* has been borne out by subsequent studies *in vivo* with Cl_2MDP (Fleisch *et al.*, in press; Russell *et al.*,1970), 25-hydroxycholecalciferol (Reynolds, 1972) and mithramycin (Reynolds, 1972). Treatment of mice with EHDP or Cl_2MDP leads to a dramatic reduction in the ability of explants to resorb *in vitro* (Figs. 6 and 7). Again Cl_2MDP is generally more effective than EHDP. The effect is present when the diphosphonate was given as shortly as 2 h before explantation, and was still present when it was given 3 days before explantation (Fig. 7). The rapid onset of action may suggest that the diphosphonates quickly adsorb onto the bone surfaces where active resorption is taking place, since they have a strong affinity for hydroxyapatite crystals (Francis, 1969; Jung *et al.*, in preparation). However, effects of the diphosphonates on cellular metabolism cannot be ruled out. In this respect, it is important to point out that *in vitro*, EHDP is more strongly bound to apatite crystals (Jung *et al.*, in preparation; Francis, personal communication) and is a more potent inhibitor of crystal dissolution than Cl_2MDP (Russell *et al.*,1970), while in the experiments in this paper and in other *in vivo* studies (Fleisch *et al.*, 1969b; Russell *et al.*, 1970; Gasser *et al.*, 1972), Cl_2MDP is more effective than EHDP. Whether this discrepancy is due to another effect of the diphosphonates than inhibiting crystal dissolution, or whether the two compounds differ in their access to the target organ, is unknown.

These results and other studies *in vivo* suggest that diphosphonates may be useful in clinical practice in diseases with increased bone destruction. This suggestion is supported by the recent results in Paget's disease (Smith *et al.*, 1971), where EHDP reduced excessive bone turnover, as judged by falls in plasma hydroxyproline and alkaline phosphatase and urinary hydroxyproline. The results of Smith *et al.* suggest that EHDP can be as effective as calcitonin (Bijvoet *et al.*, 1970; Woodhouse *et al.*, 1971) or mithramycin (for review, see Kennedy, 1970), but that it has the advantage that it is active by oral administration. It is also possible that diphosphonates will be of use in certain types of osteoporosis. Indeed, Cl_2MDP and, less so, EHDP are effective in preventing in rats the osteoporosis induced by immobilisation (Fleisch *et al.*, 1969b; Mühlbauer *et al.*, 1971; Michael *et al.*, in press). Those results and these in this paper suggest that Cl_2MDP might be even more effective than EHDP in humans. Further-

more, Cl_2MDP is for equal doses much less inhibitory of mineralisation of bone and cartilage matrix than EHDP (Gasser *et al.*, 1972; Schenk *et al.*, in preparation). Thus, its trial in humans should be worthwhile.

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