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The Effects of Diphosphonates on the Growth and Glycolysis of Connective-Tissue Cells in Culture

By DIANE K. FAST,* ROLF FELIX,† CHARLES DOWSE,‡ WILLIAM F. NEUMAN§ and HERBERT FLEISCH
Department of Pathophysiology, University of Berne, Murtenstrasse 35, 3010 *Berne, Switzerland*

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1. The effects of two diphosphonates (compounds containing a P–C–P bond), disodium dichloromethanediphosphonate and disodium 1-hydroxyethane-1,1-diphosphonate, on the metabolism of cultured rat calvaria cells, rabbit ear cartilage cells and rat skin fibroblasts were investigated. 2. The diphosphonates had no effect on the growth of cartilage cells and on the exponential growth of the calvaria cells and the fibroblasts. However, dichloromethanediphosphonate stopped the growth of the calvaria cells and the fibroblasts after the beginning of confluence, whereas the untreated cells were still growing to a certain extent. This inhibition was dose-dependent. After the drug was withdrawn, the cells recovered slowly. 1-Hydroxyethane-1,1-diphosphonate had no detectable effect on the growth of any of the cell types studied. Both diphosphonates decreased the cloning efficiency of calvaria cells and fibroblasts. 3. The K^+ content of cartilage, calvaria and skin cells was diminished only by the highest (0.25mM) concentration of dichloromethanediphosphonate. 4. Radioactive dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate were taken up linearly with time for at least 48h by calvaria cells and fibroblasts. The diphosphonate concentration in the cells depended on its concentration in the medium. 5. Both diphosphonates, in a dose-dependent fashion, markedly inhibited glycolysis, dichloromethanediphosphonate being more effective than 1-hydroxyethane-1,1-diphosphonate, at drug doses that had no effect on cell growth or cellular K^+ content. Calvaria cells were much more sensitive than cartilage cells. When cartilage cells were cultured in an N_2 atmosphere, these effects on glucose and lactate metabolism disappeared. 6. As increased acid production appears to be associated with resorption of bone, this decrease in lactate may explain why diphosphonates are effective inhibitors of bone resorption *in vivo*.

Diphosphonates, compounds containing a P–C–P bond and related to pyrophosphate but resistant to metabolic destruction, inhibit the formation and dissolution of calcium phosphate crystals *in vitro*. *In vivo*, they prevent ectopic calcification and the resorption of bone (Fleisch *et al.*, 1969, 1970; Francis *et al.*, 1969; Russell *et al.*, 1970); in some cases they also prevent the mineralization of bone (Russell *et al.*, 1973; Schenk *et al.*, 1973). Previously, these effects have been attributed to physicochemical interactions with calcium phosphate crystals. If various diphosphonates are compared, however, there is a discrepancy between their effects on crystal dissolution *in vitro* and their effects on bone resorption. For example, whereas disodium dichloromethanediphosphonate is more potent than disodium 1-hydroxyethane-1,1-diphosphonate in inhibiting bone resorption (Gasser *et al.*, 1972; Reynolds *et al.*, 1972), the latter is more potent than the former in inhibiting crystal dissolution *in vitro* (Russell *et al.*, 1970).

It seemed possible that the diphosphonates might act at least partly by altering cellular activity (Morgan *et al.*, 1973). We began investigations on the effect of two diphosphonates, dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate, on the growth and metabolism of three cell preparations grown in culture. Bone cells, ear cartilage cells and skin fibroblasts were studied to discover whether any effect we observed was general or specific.

The results of these survey studies indicate that the diphosphonates do indeed alter cellular activity in a

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

* Present address: Research Institute, McGill University–Montreal Children's Hospital, Montreal, Que. H3H 1P3, Canada.

† To whom reprint requests should be addressed.

‡ Permanent address: Department of Oral Biology, Faculty of Dentistry, University of Manitoba, Winnipeg, Man. R3E OW3, Canada.

§ Permanent address: Department of Radiation Biology, University of Rochester, Rochester, NY 14642, U.S.A.

dose-dependent fashion. Some of their effects, such as the inhibition of lactate production, may be related to the action of these drugs on bone resorption *in vivo*.

Experimental

Materials

Collagenase (*Clostridium histolyticum*, code CLS II) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; 0.25% trypsin solution, Eagle's minimum essential medium, antibiotics and foetal calf serum were from Gibco, Paisley PA3 4EP, Renfrewshire, Scotland, U.K.; deoxy[*methyl*-^{3}H]-thymidine (specific radioactivity 20Ci/mmol) was from New England Nuclear, Dreieichenhain, West Germany; Hepes was from Sigma, St. Louis, MO, U.S.A.; disodium 1-hydroxyethane-1,1-diphosphonate disodium dichloromethanediphosphonate, 1-hydroxy[^{14}C]ethane-1,1-diphosphonate and dichloro[^{14}C]methanediphosphonate (specific radioactivities 1.932Ci/mol and 0.55Ci/mol respectively) were from Procter and Gamble Co., Cincinnati, OH, U.S.A. The scintillation liquid used to determine radioactivity was composed of 80g of naphthalene, 0.5 litre of toluene, 0.4 litre of methoxyethanol (methylCellosolve) and 7g of butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole].

Methods

Preparation of cells. Cells were prepared by a modification of the method of Peck *et al.* (1964) as specified below.

(*a*) Calvaria cells. Calvariae of 1-day-old Wistar rats were used. After decapitation, the heads were kept for 1h in cold minimum essential medium with Earle's salt solution (Eagle, 1959), containing 227mg of $NaHCO_3$/l, 60μg of penicillin/ml, 100μg of streptomycin/ml, 0.25μg of fungizone/ml and 100μg of mycostatin/ml to kill bacteria and especially fungi found on rats. The calvariae were dissected and cleaned of blood vessels. Then 12–15 calvariae were shaken for 2h at 37°C in 4ml of minimum essential medium with Earle's salt solution, containing 227mg of $NaHCO_3$/litre, 60μg of penicillin/ml, 100μg of streptomycin/ml, 0.25μg of fungizone/ml (the combined penicillin, streptomycin and fungizone are subsequently called 'antibiotics') and 3mg of collagenase/ml (Worthington CLS II). The suspension containing the released cells was aspirated ten times with a Pasteur pipette to disaggregate the cells, and then decanted into a tube. Then 4ml of minimum essential medium, containing 227mg of $NaHCO_3$/litre and antibiotics, was added, and the suspension was mixed and left for 10min on ice to let the still-aggregated cells settle. The upper 6.5–7.0ml from the total of 8ml was removed and centrifuged at 350$g_{av.}$ for 7min. The pellet was resuspended in 1ml of minimum essential medium with Earle's salt solution, containing 227mg of $NaHCO_3$/litre and antibiotics, and the cells were again disaggregated by aspirating 30 times with a Pasteur pipette. If more than 15 calvariae were used the various suspensions were now combined in one tube, the total volume was made up to 8ml of minimum essential medium, and the suspension again left for 10min on ice. The upper 6.5–7.0ml was removed, diluted with minimum essential medium (containing Earle's balanced-salt solution, antibiotics and 10% foetal calf serum) to a concentration of 200000cells/ml and plated in plastic Petri dishes of 3.5cm diameter, usually at a density of 150000 cells per dish. In one case, to prolong the exponential phase, 37500cells per dish were plated. From one calvaria 200000–800000 cells were obtained. For the experiment presented in Fig. 4 24-well tissue-culture cluster dishes 3524 with a diameter of 16mm from Technomara A.G., CH-8059 Zürich, Switzerland, were used; 0.5ml of medium containing 100000 cells was added.

(*b*) Fibroblasts. For this 1-day-old Wistar rats were decapitated and the skin of the scalp was dissected after cleaning with 70% (v/v) ethanol. The skin fibroblasts were digested free and prepared exactly as for the calvariae, except that the cell suspension was filtered through a 35μm nylon mesh after digestion. Each rat yielded 130000–460000 cells.

(*c*) Ear cartilage cells. New Zealand/California rabbits, 3–4 weeks old and weighing 900–1000g, were used. The ears were left for 5min in 70% (v/v) ethanol and then rinsed with sterile distilled water. The skin was removed, the ears were rinsed with sterile water and then, to swell the fibres covering the cartilage, the ears were put in minimum essential medium with Earle's salt solution and antibiotics, containing 227mg of $NaHCO_3$/litre, for 2h. The fibres were pulled off with forceps, and the cartilage was cut into small pieces and digested with collagenase for 3h and prepared as described for the calvariae, except that the cell suspension was filtered through a 35μm nylon mesh. Some 25000 cells were plated in plastic dishes of diameter 6.0cm. From two ears 13×10^6–18×10^6 cells were obtained.

Culturing the cells. All three types of cells were cultured in a similar fashion, in minimum essential medium with Earle's balanced-salt solution, containing antibiotics and 10% foetal calf serum, and incubated at 37°C in 5% CO_2 in air. On culture day 1, the day after plating, the medium was removed, and unattached cells and debris were washed away with minimum essential medium containing Earle's salt solution. Fresh medium with or without diphosphonate was added. Subsequently the medium was changed every 3 days.

Counting the cells. After the medium was removed, 1ml of a mixture (v/v) of 5 parts of $CaCl_2$-free Hanks solution (Hanks & Wallace, 1949) plus 1 part of bicarbonate-free minimum essential medium,

containing 0.025% trypsin and 1mg of collagenase/ml, was added and the culture incubated for 3h at 37°C in 5% CO_2 in air. Then the cells were completely disaggregated by aspiration with a Pasteur pipette, diluted with Hanks solution to 50 or 100ml and counted in a Coulter counter (model industrial D, Coulter Counter Electronics, Dunstable, Beds., U.K.). After the digestion, about 15% of the cells took up dye when tested in 0.04% Trypan Blue for 2min. There was no difference between control and diphosphonate-treated cells.

The mean diameter of the cell population (calvaria cells or fibroblasts) determined with the Coulter counter was 14μm, and the calculated volume 1.44×10^{-9}cm^3 per cell.

Determination of DNA. Since the rabbit ear cartilage cells could not be disaggregated to single cells once they were confluent, the cell number could not be determined with the Coulter counter. DNA was therefore determined by the method of Burton (1956). After washing, the cells were released from the dish by incubation with 0.045% trypsin in Hanks solution at 37°C for 2h, transferred to a test tube and the final volume was adjusted to 2.5ml. Next 0.1ml of 1% (w/v) bovine serum albumin was added and then precipitated with 0.4ml of 1.875M-$HClO_4$. After standing for 30min in ice/water the solutions were centrifuged at $1000g_{av.}$ for 10min and the pellets treated as described by Leyva & Kelley (1974). In 10^6 cells $4.25\pm0.17\mu$g (mean±S.E.M.) ($n=14$) of DNA was found.

Determination of K^+. The cell monolayer was washed three times with Gey's balanced-salt solution (Gey & Gey, 1936) containing no K^+. Next 0.8ml of 3% (w/v) trichloroacetic acid was added to each dish of 3.5cm diameter (or 2.0ml to each 6cm dish) and left for 1h. The monolayer was scraped off with a 'rubber policeman' and the dish washed with 3×0.4ml (or 3×1ml) of water. The mixture was centriruged at $1000g_{av.}$ for 10min and the supernatant used for determination of the K^+ content with a flame photometer (Evans Electroselenium, Halstead, Essex, U.K.); standard solutions ranged from 0.05 to 0.25mM-KCl in 1.2% (w/v) trichloroacetic acid, and 0.25mM-diphosphonate in the standard solutions did not influence the measurement. For the calvaria cells and the fibroblasts the concentration of K^+ in the cell was calculated by dividing the K^+ content by the cell volume. The K^+ content in the rabbit ear cartilage cells was expressed as μmol of K^+/μg of DNA.

Cloning efficiency. Cells were cultured from days 1 to 7 (day 0 being the day of plating) in the presence or absence of diphosphonate. They were then released from the dish by incubation in a collagenase/trypsin mixture as described under 'Counting the cells'. The suspensions from six dishes were pooled and centrifuged at $300g_{av.}$ for 7min; the pellet was suspended in 10ml of minimum essential medium containing Earle's salt solution, antibiotics and 227mg of $NaHCO_3$/litre. The cells in a 2ml sample were counted with the Coulter counter. The cell suspension was then diluted and 300 cells were plated in plastic dishes of 8.5cm diameter. They were cultured in minimum essential medium with antibiotics plus Earle's salt solution at 37°C in 5% CO_2 in air for 6 days without changing the medium. The medium was then removed and the dishes were washed with Hanks solution. The cells were fixed with 10% formalin in Hanks solution, stained with a solution containing 1% (w/v) Fuchsin, 4.5% (w/v) phenol and 10% (v/v) ethanol, and the clones counted under the microscope. The clones had a wide range of sizes, but the size distribution was not altered by the presence of diphosphonates. The total number of clones per dish was determined.

Uptake of deoxy[methyl-^3H]thymidine. Rabbit ear cartilage cells were cultured from days 1 to 7 in the presence or absence of diphosphonate. New medium (3.5ml/dish), containing 15% foetal calf serum along with 5μCi of deoxy[*methyl*-^{3}H]thymidine (specific radioactivity 20Ci/mmol), was added to stimulate cell growth instead of the normal 10% serum. After a 24h incubation at 37°C in 5% CO_2 in air, the medium was removed and the cell layer washed once with Hanks solution containing 1mM-2′-deoxythymidine. After incubation with 1ml of 0.04% (w/v) trypsin and 1mM-2′-deoxythymidine in Hanks solution for 2h at 37°C, the cell suspension was transferred to tubes and centrifuged at $350g_{av.}$ for 7min. The supernatant was discarded and 0.5ml of cold 0.2M-$HClO_4$ added to the pellet, which was suspended and left on ice for 30min. The mixture was then centrifuged at $1000g_{av.}$ for 10min, and the pellet washed with 3×2ml of cold 0.2M-$HClO_4$ and dissolved in 0.3ml of Soluene 350 (Packard Instrument Co., Downers Grove, IL, U.S.A.). The radioactivity and the amount of total DNA were determined. The uptake was calculated as a percentage of the total radioactivity added originally to the cells and normalized for the DNA content of the dish. The blank value, obtained from cells incubated for only 15min, was less than 3% of the uptake during 24h.

Uptake of 1-hydroxy[^{14}C]ethane-1,1-diphosphonate and dichloro[^{14}C]methanediphosphonate. ^{14}C-labelled diphosphonates were added to the medium at times indicated in the text. After incubation of the cells for up to 4 days with the radioactive diphosphonate, the medium was removed, and the cell layer washed four times with a solution containing 0.145M-NaCl, 5mM-Hepes, pH7.5, and 2.5mM-non-radioactive diphosphonate. Then 0.5ml of 3% (w/v) trichloroacetic acid was added, and the monolayer was scraped off with a 'rubber policeman' and transferred to a tube. After standing for 1h at room temperature the mixture was centrifuged at $1000g_{av.}$ for 10min, and

the radioactivity of 0.3ml of the supernatant was measured. Blank values were determined by incubating the cells for only 15min at 37°C with ^{14}C-labelled diphosphonate. The concentration in the cell was calculated by dividing the mmol of diphosphonate taken up by the cell by the volume (in litres) determined with the Coulter counter.

In the first experiment testing dichloro[^{14}C]methanediphosphonate uptake, the cells were washed more extensively to ensure that the diphosphonate had been taken into the cell and was not merely adhering outside to the membrane. After the cells had been washed with 5×1ml of 0.145M-NaCl/5mM-Hepes, pH7.5, half of the dishes were treated with collagenase/trypsin to release the cells. These cell suspensions were centrifuged at $350g_{av.}$ for 7min, and the pellets resuspended and washed twice more. As shown in Table 1, there was an increase in radioactivity in the washing water after the cells had been released from the dish by enzyme digestion, perhaps through release of diphosphonate from the cell interior. Since at the fifth washing only a small amount of radioactivity was in the washing water, the radioactivity in the cells was determined after four washings in all future experiments.

Lactate production and glucose consumption. Fresh medium with 10% foetal calf serum was added and the cells were incubated at 37°C in 5% CO_2 in air for 16h. The medium was then removed and kept frozen until the lactate and glucose concentrations were measured in an $HClO_4$ extract. Lactate dehydrogenase (for lactate) (Bergmeyer, 1965*b*) and a mixture of hexokinase and glucose dehydrogenase (for glucose) (Bergmeyer, 1965*a*) were used for the determinations. The presence of the diphosphonates did not influence the determinations. Lactate production and glucose consumption were almost linear with time during this period. There was about a 15% decrease from linearity after 16h.

Lactate dehydrogenase. This was measured as described by Bergmeyer (1965*c*).

Table 1. *Dichloro[^{14}C]methanediphosphonate found in the wash medium and in the cells*

Absolute values and the percentage of total (348100 c.p.m.) are given. The medium was removed, the monolayers were washed five times, and the radioactivity of the solutions was determined. To half of the dishes 3% trichloroacetic acid was added, the monolayers were scraped off with a 'rubber policeman', the mixture was centrifuged and the radioactivity in the supernatant measured (=cell content I). In the other half of the dishes the cells were released with collagenase/trypsin solution and the suspension was centrifuged. The radioactivity in the supernatant was the sixth washing. The pellet was resuspended and washed twice more, which gave the seventh and eighth washings. To the final pellet 3% trichloroacetic acid was added, and after centrifugation the radioactivity of a sample of the supernatant was determined (=cell content II).

Solution	Radioactivity found (c.p.m.) [±S.E.M. (*n*)]	Percentage of total (%)
Medium	334675±2245 (9)	96.1
1st washing	10493± 428 (9)	3.0
2nd washing	1035± 72 (9)	0.30
3rd washing	132± 11 (9)	0.038
4th washing	105± 9 (9)	0.030
5th washing	48± 5 (9)	0.014
Cell content I	1594± 31 (4)	0.46
6th washing	273± 9 (5)	0.078
7th washing	114± 8 (5)	0.033
8th washing	111± 17 (5)	0.032
Cell content II	1116± 33 (5)	0.32

Results

Effects of diphosphonates on cell growth

Neither dichloromethanediphosphonate nor 1-hydroxyethane-1,1-diphosphonate had any detectable effect on the growth of cartilage cells (Fig. 1). Throughout the exponential phase and the subsequent plateau phase of growth the cell number, as indicated by DNA content, was unaffected by 0.25mM of either drug.

Although neither diphosphonate exerted a detectable effect on the growth of cartilage cells as measured by DNA content, when the incorporation of labelled thymidine was examined, a small but significant

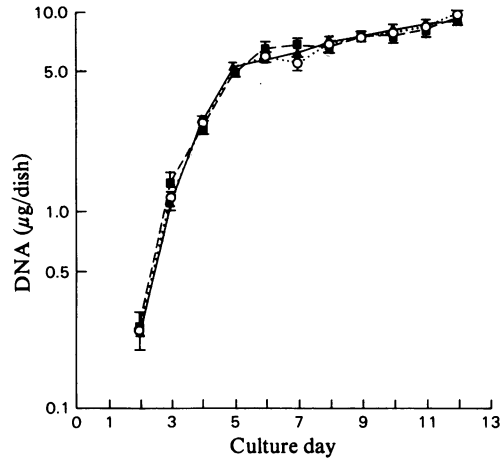

Fig. 1. *Growth curve of rabbit ear cartilage cells in the presence and absence of diphosphonates*

For this 25000 cells were plated in 6cm-diameter dishes on day 0. The disphophonates were added on day 1. The DNA content was determined every day from day 2 to 13 in three separate dishes. The mean ±S.E.M. is given. ○, Control; ▲, +0.25mM-1-hydroxyethane-1,1-diphosphonate; ■, +0.25mM-dichloromethanediphosphonate.

decrease, at least in one experiment, was caused by dichloromethanediphosphonate, as shown in Table 2.

Fibroblasts, and especially calvaria cells, however, were more sensitive. As shown for calvaria cells in Fig. 2, this sensitivity was not expressed during the exponential phase of growth, but only as the cells approached confluence. It should be emphasized that after reaching confluence the untreated cells did not stop growing completely (Figs. 2, 3 and 4). However, 0.25mM-dichloromethanediphosphonate decreased the cell number compared with the control after the beginning of the plateau phase (culture day 5). That this action, on calvaria cells at least, was dose-dependent is shown in Table 3. At 0.25mM-dichloromethanediphosphonate the number of cells in the plateau phase was halved; the effect virtually disappeared at a concentration of 0.0025mM. 1-Hydroxyethane-1,1-diphosphonate had no detectable effect on either cell type.

Table 2. *Effect of diphosphonates on thymidine incorporation by cartilage cells*

Rabbit ear cartilage cells were grown in the presence or absence of 0.25mM-1-hydroxyethane-1,1-diphosphonate or 0.25mM-dichloromethanediphosphonate. The mean uptake of deoxy[*methyl*-^{3}H]thymidine by cells in five dishes (±1 S.E.M.) is given, expressed as percentage of control. The uptake in the control was 0.69% of the total radioactivity added to the dish (5μCi) in Expt. I and 0.38% in Expt. II.

	Incorporation (% of controls)	
	Expt. I	Expt. II
Control	100 ±1.7	100 ±2.6
1-Hydroxyethane-1,1-diphosphonate (0.25mM)	93.3±2.7	103 ±2.2
Dichloromethane-diphosphonate (0.25mM)	86.1±2.6*	93.5±2.9

* Student's *t* test of significance, $P<0.025$.

This drug effect of decreasing the cell number was observed only at confluence, when the cells would be undergoing contact inhibition. It was important therefore to investigate (1) whether the effect was dependent on cell concentration, (2) if exposure to diphosphonate before confluence was necessary and (3) whether the inhibition, once expressed, was permanent. To examine the first problem, fibroblasts were plated over a range of initial cell concentrations and grown for various time periods in the presence or absence of 0.25mM-dichloromethanediphosphonate

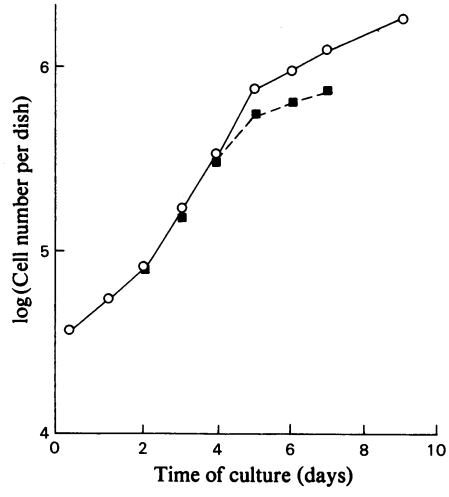

Fig. 2. *Growth curve of rat calvaria cells in the presence and absence of* 0.25 *mM-dichloromethanediphosphonate*

For this 37500 calvaria cells were plated in 3.5cm-diameter dishes on day 0; 0.25mM-dichloromethanediphosphonate was added on day 1. The cell number of three separate dishes was determined every day. S.E.M. values are not drawn, because they are smaller than the symbols. ○, Control; ■, +0.25mM-dichloromethanediphosphonate.

Table 3. *Inhibition of cell growth by diphosphonates*

For this 150000 cells were plated in 3.5cm dishes. The diphosphonates were added on day 1. The cell numbers in the controls were [means±1 S.E.M. (*n*)]: calvaria cells at day 7 (plateau phase): 2103500±116500 (15), at day 10: 2969000 ±80000 (7); fibroblasts at day 7: 1677200±102200 (4), at day 10: 1984000±155500 (5). Results are means±S.E.M. for the numbers of experiments in parentheses.

	Inhibition (%)				
	1-Hydroxyethane-1,1-diphosphonate		Dichloromethanediphosphonate		
Culture	0.25mM	0.025mM	0.25mM	0.025mM	0.0025mM
Calvaria 7 days	5.3± 3.6 (12)	1.0±3.4 (8)	50.5±2.5 (13)	14.2±2.9 (11)	7.8±3.7 (7)
10 days	1.4± 1.2 (6)	—	57.2±3.6 (6)	33.6 (1)	—
Fibroblast 7 days	−9.8± 3.8 (3)	—	38.3±4.4 (4)	—	—
10 days	−8.9±12.6 (5)	—	53.5±3.7 (4)	—	—

(Table 4). The greater the final cell density, which was dependent both on culture time and the original cell density, the greater was the inhibition of cell growth by dichloromethanediphosphonate.

Secondly, to study whether exposure to diphosphonate before confluence was necessary, calvaria cells were cultured for 7 days and 0.25mM-dichloromethanediphosphonate was added to half the dishes on day 8 (Fig. 3). There was little effect for 24h, but by 48h there was a decrease in cell number in the diphosphonate-treated culture compared with controls. The decrease was 5, 17, 16, 42 and 63% on days 9, 10, 11, 14 and 16 respectively.

Thirdly, to test whether the inhibition, once expressed, was permanent, calvaria cell cultures were grown in the presence or absence of 0.25mM-dichloromethanediphosphonate up to day 7. After this time diphosphonate treatment was stopped on one series of dishes and the growth of the three cultures compared. These results are presented in Fig. 4. In the dichloromethanediphosphonate-treated culture the cell number stayed constant from day 4 to day 22, whereas in the control the cell number still increased to a certain extent, although the cells had reached confluence. After withdrawal of the compound at day 7, the cells started to grow again and at day 22 almost the same cell number was reached as in the control.

The effects of the two diphosphonates on cloning efficiency are shown in Table 5. Both drugs sharply decreased the number of clones produced by calvaria cells and by fibroblasts, the effect being dose-dependent. Again, dichloromethanediphosphonate was slightly more effective than 1-hydroxyethane-1,1-diphosphonate.

Effects on K^+ content of the cells, and lactate dehydrogenase of the medium

As a general index of viability, the K^+ content of the cells was determined. When the effect of the diphosphonates on K^+ content was examined (Table 6), it was found that only at the highest concentration of dichloromethanediphosphonate and only late in

Table 4. *Effect of cell density on growth inhibition of fibroblasts by 0.25 mM-dichloromethanediphosphonate*

Fibroblasts were plated at different cell densities and grown in the presence or absence of 0.25mM-dichloromethanediphosphonate for various times. The values are the means of duplicates, expressed as percentage inhibition of the controls (also in duplicates).

	Inhibition (%)			
	Cells originally cultured per dish ...			
Day of culture	75000	150000	300000	450000
2	0	0	0	0
3	14	14	10	13
4	19	13	18	27
5	23	28	34	36
7	30	38	55	65
11	55	62	71	72

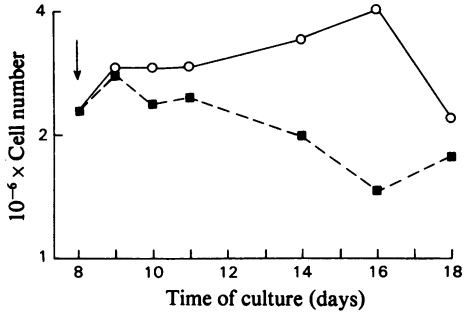

Fig. 3. *Effect of dichloromethanediphosphonate added in the plateau phase*

Calvaria cells were grown in the absence of diphosphonates. At day 8, 0.25mM-dichloromethanediphosphonate was added to half the cultures. ○, Control; ■, +0.25mM-dichloromethanediphosphonate. Each symbol represents the mean of three dishes; 1 S.E.M. was less than 2.5%, except at control day 14, where it was 5%.

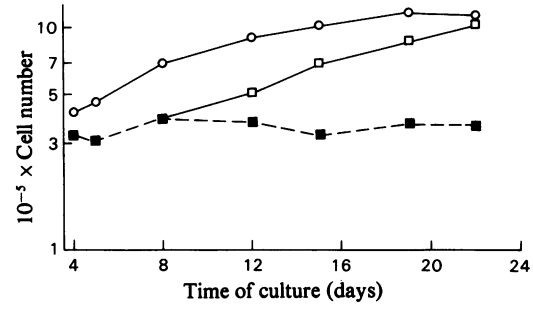

Fig. 4. *Growth curve of calvaria cells after removal of dichloromethanediphosphonate*

Calvaria cells were grown from day 1 to 22 in the presence or absence of 0.25mM-dichloromethanediphosphonate. In a third series of dishes the diphosphonate was withdrawn at day 7. The values drawn on a log scale are the means for four dishes. The S.E.M. values were always below 4%. ○, Control; ■, +0.25mM-dichloromethanediphosphonate; □, +0.25mM-dichloromethanediphosphonate from day 1 to 7, no diphosphonates from day 8 to 22. In this experiment, instead of 3.5cm-diameter dishes, dishes with 16mm diameter were used, to which 10^5 cells were seeded.

Table 5. *Effect of diphosphonates on cloning efficiency*

Values are the means±S.E.M. for four dishes. The average control culture gave 52.2±3.6 clones for 300 cells cultured in an 8.5cm dish.

	No. of clones (% of control)				
	1-Hydroxyethane-1,1-diphosphonate		Dichloromethanediphosphonate		
	0.25mM	0.025mM	0.25mM	0.025mM	0.0025mM
Calvaria (Expt. I)	44.5± 1.0*	78± 2.1†	42.5±2.8*	66 ± 5.1*	92±6.5
(Expt. II)	66 ± 4.1*	93±11	69 ±4.8*	81.5±11	93±4.5
Fibroblasts	86 ±11		35 ±5.9*		

* Significantly less than controls, $P<0.01$.
† Significantly less than controls, $P<0.02$.

Table 6. *Effect of diphosphonates on K^+ content of culture cells*

The mean K^+ content of control cells was 161 mM, 110 mM and 70 nmol/μg of DNA for the calvaria, fibroblast and cartilage cells respectively. The number of dishes are in parentheses. The means ± S.E.M. are given.

	K^+ content (% of controls)				
	1-Hydroxyethane-1,1-diphosphonate		Dichloromethanediphosphonate		
	0.25mM	0.025mM	0.25mM	0.025mM	0.0025mM
Calvaria 7 days	93±6.8 (7)	95±2.6 (9)	78±8.0* (7)	99±1.2 (10)	94±1.2 (10)
10 days	90±1.0* (5)	—	68±2.6* (5)	—	—
Fibroblasts 7 days	115±3.1† (7)	—	109±4.7 (8)	—	—
10 days (Expt. I)	135±1.9* (4)	—	56±5.9* (4)	—	—
10 days (Expt. II)	118±1.2* (4)	—	87±3.8 (5)	—	—
Cartilage cells 7 days (Expt. I)	96±1.2 (7)	—	76±1.3* (7)	—	—
7 days (Expt. II)	99±2.9 (5)	—	88±1.5* (5)	—	—

* Significantly different from control, $P<0.01$.
† Significantly different from control, $P<0.05$.

Table 7. *Diphosphonate concentrations in the cells*

Calvaria cells or fibroblasts were grown from day 1 in the presence of non-radioactive 0.25 or 0.025mM-diphosphonate. During the time indicated in the Table ^{14}C-labelled diphosphonate was added to the culture. The concentrations in the cells were calculated as indicated under 'Methods'. The means of individual dishes±1 S.E.M. (*n*) are given.

Expt. no.	Diphosphonate concentration in medium (mM)	Cell type	Time of exposure to ^{14}C-labelled diphosphonates (days)	Concn. in the cell (mmol/litre)	
				1-Hydroxyethane-1,1-diphosphonate	Dichloromethane-diphosphonate
I	0.25	Calvaria	2– 4	—	0.19 ±0.004 (4)
	0.25	Calvaria	8–10	—	0.58 ±0.01 (5)
II	0.25	Calvaria	2– 4	—	0.37 ±0.007 (5)
	0.25	Calvaria	7– 9	—	0.50 ±0.015 (4)
III	0.25	Calvaria	7– 9	0.37 ±0.005 (4)	0.50 ±0.01 (4)
	0.25	Calvaria	7–11	0.53 ±0.03 (4)	0.74 ±0.01 (4)
IV	0.25	Fibroblasts	2– 4	0.46 ±0.002 (3)	0.50 ±0.02 (4)
	0.25	Fibroblasts	9–11	0.61 ±0.03 (3)	0.58 ±0.01 (3)
V	0.025	Calvaria	6– 8	0.070±0.006 (4)	0.030±0.005 (4)
VI	0.025	Calvaria	7– 9	0.073±0.007 (4)	0.041±0.004 (4)

the plateau phase of growth did the drugs appreciably alter the K^+ content. Only dichloromethanediphosphonate caused a significant decrease in all three cell types. 1-Hydroxyethane-1,1-diphosphonate had very little effect, although in fibroblasts the K^+ content was increased. Lactate dehydrogenase in the medium, added to the cells from day 4 to day 7, was measured, and expressed as a percentage of the enzyme activity found in the cells. Compared with the controls, the values were increased by 3.3 and −0.5% for 1-hydroxyethane-1,1-diphosphonate-treated cells and 13.2 and 5.9% for dichloromethanediphosphonate-treated cells.

Uptake of diphosphonates by cells

To test whether or not the diphosphonates were taken up by the cells, labelled dichloromethanediphosphonate or 1-hydroxyethane-1,1-diphosphonate was added to calvaria cells or fibroblasts that had been grown in the presence of non-radioactive diphosphonate. Table 7 shows that the diphosphonates were taken up by the cells and that the concentration of diphosphonate in the cells was dependent on the concentration in the medium. At 0.25mM both diphosphonates were taken up to the same degree, but at 0.025mM less dichloromethanediphosphonate than 1-hydroxyethane-1,1-diphosphonate was taken up. The concentration of radioactive diphosphonate in the cells appeared higher when the uptake was measured in the plateau rather than in the exponential phase (Table 7, Expts. I, II and IV). If the cells were exposed to labelled diphosphonate for 4 days instead of for 2 days, the concentration in the cells was increased (Expt. III). The fibroblasts took up the diphosphonates as well as did the calvaria cells.

To investigate the rate of uptake, the incorporation of labelled diphosphonates by calvaria cells that had not previously been exposed to diphosphonates was measured as a function of time. As shown in Fig. 5, there was a continuous uptake for the duration of the experiment (48h).

Effects on glycolysis

One of the striking features of bone cells and connective-tissue cells in general is their production of lactate. Although the Pasteur effect, the inhibition of glycolysis by oxygen, still occurs, these cells produce large amounts of lactate even in the presence of oxygen (Borle *et al.*, 1960). To study whether the

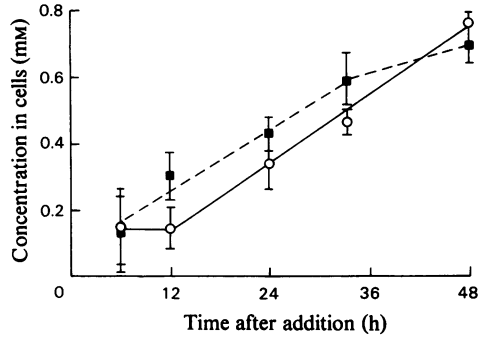

Fig. 5. *Time course of* 1-*hydroxyethane*-1,1-*diphosphonate and dichloromethanediphosphonate uptake*
After growth for 6 days in the absence of diphosphonate, 0.25mM-1-hydroxy[^{14}C]ethane-1,1-diphosphonate (○) or 0.25mM-dichloro[^{14}C]methanediphosphonate (■) was added to the culture, and the radioactivity in the cells was measured after 0, 6, 12, 24, 36 and 48h. The concentrations of radioactive diphosphonates in the cell were calculated as described under 'Methods'. The means±S.E.M. for four dishes are given.

Table 8. *Inhibition of lactate production and glucose consumption by diphosphonates*
Rat calvaria cells or rabbit ear cartilage cells were grown from days 1 to 7 in the presence or absence of dichloromethanediphosphonate or 1-hydroxyethane-1,1-diphosphonate. Lactate production and glucose consumption were measured from day 7 to 8 (16h). The results are expressed as percentage of the control and are the means±S.E.M. for three or four individual experiments, except where only one experiment was done. For each experiment three or four individual dishes were used for the measurements. In the control the calvaria cells produced 3.45±0.52 (4)μmol of lactate/10^6 cells and consumed 2.25±0.35 (4)μmol of glucose/10^6 cells, the rabbit ear cartilage cells produced 2.12±0.19 (4) μmol of lactate/μg of DNA and consumed 1.23±0.14 (4)μmol of glucose/μg of DNA.

	Lactate production (% of control)		Glucose consumption (% of control)	
Diphosphonate concentration	Calvaria cells	Cartilage cells	Calvaria cells	Cartilage cells
Dichloromethanediphosphonate 0.0025mM	85.5 (1)	—	89.9 (1)	—
0.025mM	33.6± 7.2 (4)	92.7 (1)	42.2±8.3 (4)	92.2 (1)
0.25mM	3.6±11 (4)	19.8±6.1 (4)	−2.4±7.7 (4)	49.2± 7.7 (4)
1-Hydroxyethane-1,1-diphosphonate 0.025mM	72.6 (1)	—	76.8 (1)	—
0.25mM	44.8± 7.5 (4)	86.9±8.4 (4)	50.4±4.0 (4)	91.4±17.9 (4)

Table 9. *Effect of diphosphonates on the control of anaerobic glycolysis*

Rabbit ear cartilage cells were grown for 1 to 7 days in the presence or absence of 0.25mM-1-hydroxyethane-1,1-diphosphonate or 0.25mM-dichloromethanediphosphonate. Lactate production and glucose consumption were measured after 16h (from day 7 to 8) from cells in either 5% CO_2/95% air or in 5% CO_2/95% N_2. The means±S.E.M. of three dishes are given. The DNA contents in all dishes were almost the same.

Diphosphonate	Lactate production (μmol/dish)		Glucose consumption (μmol/dish)		Ratio lactate/glucose	
	5% CO_2/air	5% CO_2/N_2	5% CO_2/air	5% CO_2/N_2	5% CO_2/air	5% CO_2/N_2
None	21.7±1.1	38.4±2.1	13.1±0.6	19.1±0.5	1.66	2.00
1-Hydroxyethane-1,1-diphosphonate	17.7±1.3	39.4±2.4	12.1±0.6	19.3±0.5	1.46	2.04
Dichloromethanediphosphonate	4.6±1.0	32.9±0.8	5.6±0.5	17.1±0.6	0.82	1.92

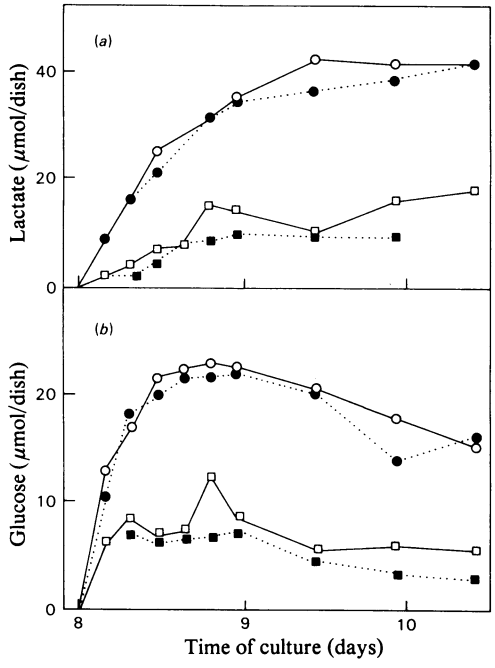

Fig. 6. *Time course of the dichloromethanediphosphonate effect on lactate production and glucose consumption in rabbit ear cartilage cells*

The culture period was divided into two periods: (i) 1–7 days and (ii) 8–11 days. Four combinations of exposure to 0.25mM-dichloromethanediphosphonate were examined: ○, days 1–7 no drug, days 8–11 no drug; ●, days 1–7 no drug, days 8–11 0.25mM-drug; □, days 1–7 0.25mM-drug, days 8–11 no drug; ■, days 1–7 0.25mM-drug, days 8–11 0.25mM-drug. The glucose consumption (*a*) and the lactate production (*b*) were studied as a function of time after 8 days of culture. Each point is the value of a single determination.

diphosphonates altered cellular metabolism, their possible effects on glucose consumption and lactate production were investigated. Table 8 shows that there were dramatic changes in both these metabolic parameters for all three cell types studied. As in experiments described above, dichloromethanediphosphonate was more potent than 1-hydroxyethane-1,1-diphosphonate, but both agents markedly decreased the consumption of glucose and the production of lactate. Moreover, these effects were seen at doses that had no detectable effects on cell growth or on cellular K^+ content.

To determine whether this represented a change in enzymic profile or merely a regulatory shift, i.e. the expression of the usual Pasteur effect, cartilage cells were tested for lactate production and glucose consumption both in air/CO_2 and N_2/CO_2. These results are summarized in Table 9. As before, in air the diphosphonates decreased both lactate production and glucose consumption. However, in an N_2 atmosphere, the effects disappeared. Thus the enzymic machinery for a fully glycolytic rate of metabolism seemed to be present in the treated cells.

To examine whether these metabolic effects depended on when the diphosphonate was added or for how long it was present, cartilage cells were grown in the presence or absence of 0.25mM-dichloromethanediphosphonate for 7 days. After this time, half the cultures grown in the absence of dichloromethanediphosphonate received diphosphonate, and half those grown in the presence of dichloromethanediphosphonate were given diphosphonate-free medium. The glucose consumption and lactate production from day 8 to 11 are shown in Fig. 6. Dichloromethanediphosphonate, when given only from day 8 to 11, was without effect, but, after exposure to the drug during the first 7 days of culture, the cartilage cells continued to show decreased glucose consumption and lactate production even after the drug was withdrawn.

Discussion

At this stage of our knowledge, it is difficult to interpret the effect of diphosphonates on cell growth. All three cell preparations were to some extent mix-

tures of cell types, the calvaria cells being the most complex mixtures. The cartilage-cell mixture was the least affected, and the calvaria cells were most sensitive to the growth inhibition caused by the diphosphonates. Whether this effect is due to a selective inhibition of certain cell types or to a general effect on all cells in the mixed cultures is still uncertain.

However, truly significant growth effects were observed predominantly at the highest concentrations of dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate in cells exposed for prolonged periods and in their plateau phase of growth. Similarly, the K^+ content of cells, which is a very sensitive indicator of cell viability, was unaffected at the lower concentrations of diphosphonates. Thus the very low toxicity of the diphosphonates observed *in vivo* (Nolen & Buehler, 1971) was confirmed in the cultured cells *in vitro*.

The diphosphonates are bound strongly to apatite crystals (Jung *et al.*, 1973), and the bone mineral therefore adsorbs nearly all of the diphosphonate delivered to the skeleton by the circulation. In the kidney, the diphosphonates are excreted very efficiently, actually showing a clearance higher than that of inulin (Troehler *et al.*, 1975). Thus the combination of sequestration by bone and excretion by the kidney results in a very rapid clearance and a very low circulating concentration of the diphosphonates, even after chronic administration of the drugs.

A low plasma concentration, however, does not mean a low diphosphonate concentration in the fluid surrounding the bone cells. Indeed, when the bone mineral is solubilized, the sequestered drug would be released, presumably in a very localized area, so that bone cells, and especially those engaged in osteoclastic activity, are likely to be exposed chronically to appreciable concentrations of diphosphonates *in vivo*. These concentrations are unknown, but it is not unreasonable to assume that they could lie in the range found effective in this study. Interestingly, fibroblasts and calvaria cells were more sensitive to the same exposure to the diphosphonates than were cartilage cells. This mechanism of sequestration and local release in bone and the greater sensitivity of bone cells could explain why the effects of diphosphonates *in vivo* have until now been found only on mineralized tissues.

Further support for this concept is given by the results of the studies of uptake of labelled diphosphonate. The uptake by cells was very slow, linear with time up to at least 48h, and, within the limits of the experiment, apparently irreversible. Thus the cell content of either diphosphonate increased with extracellular concentration and with time of exposure. Only cells exposed to appreciable concentrations for long periods of time can accumulate significant quantities of diphosphonates. Presumably only selected cells in the skeleton would meet this requirement *in vivo*. The slow uptake of diphosphonates correlates with observations *in vivo*. Walton *et al.* (1975) found that in man plasma phosphate concentrations increased during 1-hydroxyethane-1,1-diphosphonate treatment, but reached its maximum value only after 2–3 weeks' administration.

Almost all of the glucose is transformed into lactate by the cultured cells studied, and perhaps the most significant findings of these survey studies were the changes in glycolysis induced by the diphosphonates. A clear inhibition of lactate production with a concomitant sparing of glucose utilization was observed at concentrations of diphosphonates that had no effect on cell growth or K^+ content. Although these changes persisted after removal of the drug from the medium, the altered metabolic pattern appeared to be only a matter of internal regulation. Thus, when the inhibited cells were placed in an N_2 atmosphere, they adapted as well as untreated cells and showed a full normal glycolytic capacity. The site of action of the diphosphonates is unknown, but it has been shown that diphosphonates given *in vivo* or *in vitro* delay the release of Ca^{2+} from mitochondria (Guilland *et al.*, 1974; Guilland & Fleisch, 1974). In diphosphonate-treated cartilage cells, the ratio of lactate to glucose decreased (Table 9). This could suggest that the diphosphonates stimulate directly the tricarboxylic acid cycle in comparison with glycolysis. On the other hand, no change in the ratio of lactate to glucose was observed in calvaria cells treated with diphosphonates. Another possible mechanism could be the action of the diphosphonates on phosphofructokinase, which is the main regulatory enzyme of glycolysis. The measurement of glycolytic intermediates might give an answer to this question.

Lactate production by bone cells has long been thought to be intimately related to calcium homoeostasis and especially to bone resorption (Borle *et al.*, 1960; Vaes, 1968). Thus the inhibition of aerobic glycolysis observed in the present studies may well be an important part of the diphosphonate inhibition of resorption *in vivo*. The glycolytic inhibition by dichloromethanediphosphonate was much greater than that by 1-hydroxyethane-1,1-diphosphonate, the biological effectiveness of these compounds *in vivo* (Russell *et al.*, 1970; Gasser *et al.*, 1972; Reynolds *et al.*, 1972) correlating well with their actions on cell cultures *in vitro*.

In conclusion, the effectiveness of the diphosphonates may now be attributed to the combination of two properties: (1) their physicochemical propensity to concentrate selectively in bone, and (2) their ability to inhibit lactate production by cells (Morgan *et al.*, 1973). The diphosphonates have already proved useful in the management of Paget's disease (a disease, where bone destruction and turnover is increased) (Altman *et al.*, 1973; Gunčaga *et al.*, 1974; Russell *et al.*, 1974). By screening for selective bone

deposition and lactate inhibition, it may be possible to develop even more effective drugs for the treatment of resorptive disorders of bone.

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