Increase in Alkaline Phosphatase Activity in Calvaria Cells Cultured with Diphosphonates

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1. Dichloromethanediphosphonate and to a lesser degree 1-hydroxyethane-1,1-diphosphonate, two compounds characterized by a P–C–P bond, increased the alkaline phosphatase activity of cultured rat calvaria cells up to 30 times in a dose-dependent fashion. 2. Both diphosphonates also slightly inhibited the protein synthesis in these cells. 3. Thymidine, an inhibitor of cell division, did not inhibit the induction of the enzyme, indicating that the increase in enzyme activity was not due to the formation of a specific population of cells with high alkaline phosphatase activity. 4. The effect on alkaline phosphatase was suppressed by the addition of cycloheximide, an inhibitor of protein synthesis. 5. After subculturing the stimulated cells in medium without diphosphonates, the enzyme activity fell almost to the control value. 6. Bovine parathyrin diminished the enzyme activity of the control cells and the cells treated with dichloromethanediphosphonate; however, at high concentration the effect of parathyrin was greater on the diphosphonate-treated cells than on the control cells. 7. The electrophoretic behaviour, heat inactivation, inhibition by bromotetramisole or by phenylalanine, and the K_m value of the induced enzyme were identical with that of the control enzyme.

Alkaline phosphatase activity can be increased in many cultured cell types by hyperosmolarity (Nitowsky *et al.*, 1963), by substrates such as phenyl phosphate or β -glycerophosphate (Cox & Pontecorvo, 1961), and by cortisol analogues (Cox & MacLeod, 1961), aliphatic monocarboxylates (Griffin *et al.*, 1974; Deutsch *et al.*, 1977), dimethyl DL-2,3-distearoyloxypropyl-2'-hydroxyethylammonium acetate (Melnykovich & Lopez, 1977), methotrexate or actinomycin D (Speeg *et al.*, 1977), 5-bromo-2'-deoxyuridine, 1- β -D-arabinofuranosylcytosine, mitomycin C or phlebotomycin (Koyama & Ono, 1972; Chou & Robinson, 1977a,b) and by cyclic nucleotides (Koyama *et al.*, 1972; Griffin *et al.*, 1974; Chou & Robinson, 1977b; Skřivanová & Hynie, 1977). As reported in the present paper dichloromethanediphosphonate increases alkaline phosphatase activity in cultured calvaria cells.

Diphosphonates are compounds that contain a P–C–P bond and are thus related to pyrophosphate but are resistant to metabolic destruction. They inhibit bone mineral formation and mineral dissolution both *in vitro* and *in vivo* (Russell & Fleisch, 1975). The effects *in vivo* have been attributed to physicochemical interactions with calcium phosphate crystals, but there have also been observations suggesting an interaction of these compounds with cellular metabolism. For example, dichloromethanediphosphonate inhibits the lactate production stimulated by parathyrin in cultured mouse calvaria (Morgan *et al.*, 1973). Furthermore 1-hydroxyethane-

1,1-diphosphonate and dichloromethanediphosphonate inhibit the glycolysis in cultured calvaria cells, fibroblasts and cartilage cells (Fast *et al.*, 1978). It is possible that at least part of the effect *in vivo*, especially the inhibition of bone resorption, is due to a direct effect on cell metabolism. Whether the reported effect on alkaline phosphatase is in some way also relevant to the calcium phosphate metabolism cannot be answered.

Materials

Collagenase (*Clostridium histolyticum*; code CLS II) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; 0.25% trypsin solution and foetal calf serum were from Gibco, Paisley PA3 4EP, Renfrewshire, Scotland, U.K.; Eagle's minimum essential medium was from Seromed G.m.b.H., 8000 München 71, West Germany; bovine parathyrin extract [248 units/mg where 1 unit is defined as described by Munson (1961)] was from Inolex Corp., Chicago, IL, U.S.A. Disodium 1-hydroxyethane-1,1-diphosphonate and disodium dichloromethanediphosphonate were obtained from Procter and Gamble Co., Cincinnati, OH, U.S.A.; L-[4,5-³H]lysine was from New England Nuclear, Dreieichenhain, West Germany; L-phenylalanine was from Sigma, St. Louis, MO, U.S.A.; (+)- and (–)-*p*-bromotetramisole oxalate were from Aldrich, Europe Division, 2340 Beerse, Belgium; Fast Blue, Amido Black, cycloheximide and 2-naphthyl phosphate were

from Fluka A.G., Buchs, Switzerland; colchicine was from Sandoz A.G., Basel, Switzerland; actinomycin D was from Calbiochem, San Diego, CA, U.S.A.; Triton X-100 was from Christ A.G., Aesch, Switzerland; butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] was from Ciba, Basel, Switzerland. All the other reagents were analytical grade and were obtained from Merck A.G., Darmstadt, West Germany.

Methods

Cell culture

The procedure described by Fast *et al.* (1978) was employed with a few alterations. Briefly, calvaria from 1-day-old Wistar rats were digested with collagenase and the liberated cells were cultured in 24-well tissue culture cluster-dishes 3524 (diameter 16mm; Costar, Cambridge, MA, U.S.A.) in CO₂/air (1:19) in 0.5ml of minimum essential medium (Eagle, 1959) containing 10% (v/v) foetal calf serum. When larger amounts of cells were needed, Petri dishes with a diameter of 3.5cm (Corning Glass Works, Corning, NY, U.S.A.) containing 1.5ml of medium were used. The initial density of the cell suspension was 200000 cells per ml, so that 100000 and 300000 cells respectively were plated per dish. The cell number at the end of the culture period was determined with a Coulter Counter (Fast *et al.*, 1978). In the experiments in which the cells were subcultured, the cells were released as described above and replated at a cell density of 200000 cells/ml (Fast *et al.*, 1978).

Protein synthesis

L-[4,5-³H]Lysine (7.5μCi; specific radioactivity 63.1 Ci/mmol) was added to each 1ml of minimum essential medium containing calf serum and the cells were incubated for 24h. The medium was then discarded and the cells were washed twice with 0.5ml of cold Hanks solution (Hanks & Wallace, 1949). After the addition of 0.5ml of cold 5% (w/v) trichloroacetic acid the cells were scraped off with a rubber 'policeman'. The fragments were then broken to a fine suspension by sonication (Sonifier Branson B-12, Branson Sonic Power Co., Danbury, CO, U.S.A.) and the contents were transferred to a centrifuge tube. The dishes were washed with 3 × 0.5ml of cold trichloroacetic acid and the washings were also added to the tube. The suspension was centrifuged at 1500g_{av.} for 15min. After the supernatant had been decanted, 100μl of a solution of bovine serum albumin (1mg/ml) and 2ml of cold trichloroacetic acid were added to the pellet, which was then suspended in an ultrasound bath (Grieshaber and Co., Zurich, Switzerland; model 100S) and

centrifuged at 2000g_{av.} for 15min. This washing with 2ml of trichloroacetic acid was done 4 times. The final pellet was then transferred with 0.35ml of water to a scintillation vial along with two 0.2ml water rinses of the centrifuge tube. These samples were mixed with 10ml of scintillator fluid (0.25litre of Triton X-100, 0.75litre of toluene, 7g of butyl-PBD and 80g of naphthalene) and counted for radioactivity in a Packard liquid-scintillation counter.

Extraction of enzyme activity

At the end of the culture period, the medium was aspirated, the cell layer was washed 3 times with cold Hanks solution (0.5ml for 16mm dishes, 1.5ml for 35mm dishes) and the cells were then frozen in the dish.

For determinations of enzyme activity, 0.2ml of 0.1% (w/v) Triton X-100 in water was added to each dish, the suspension was then frozen and thawed three times, sonicated for 10s and then directly used for the enzyme assays.

For heat inactivation, inhibition and electrophoresis studies the enzyme was extracted with butan-1-ol or 0.1% (w/v) Triton X-100.

Extraction with butan-1-ol. The cells were scraped from the dish with a rubber 'policeman' (15 control dishes or 15 dishes treated with dichloromethanediphosphonate) and transferred with 1ml of water/dish to a stoppered glass flask. The same volume of butan-1-ol (15ml) was added and the mixture shaken for 1min and then stirred for 30min. After centrifugation at 12000g_{av.} for 5min the aqueous phase was separated, dialysed against 5mM-Tris/HCl, pH 8.5, and concentrated on an Amicon Filter UM-10 (mol.wt. cut-off 10000). The enzyme solutions were kept frozen until they were assayed.

Extraction with Triton X-100. Triton X-100 [0.4ml of a 0.1% (w/v) solution] was added to each dish (15 control dishes and 15 dishes treated with dichloromethanediphosphonate). The cell layer was scraped off with a rubber 'policeman,' collected and homogenized with a Virtis homogenizer (model 60; Virtis Co., Gardiner, NY, U.S.A.) at 30000rev./min for 30s. The homogenates were kept frozen until they were used for the assay.

Enzyme assays

Alkaline phosphatase. The enzyme activity was measured as described by Morgan *et al.* (1973): 10–50μl of solution with enzyme activity was incubated in 0.5ml of medium. After 15 or 30min the enzyme reaction was stopped by adding 2.0ml of 1M-NaOH.

Acid phosphatase. The enzyme activity was measured as described by Barrett (1972): 50μl of solution with enzyme activity was incubated with

0.4 ml of 0.2 M-sodium acetate buffer, pH 5.0, at 37°C for 10 min to destroy glucose 6-phosphatase. The acid phosphatase reaction was initiated by adding 0.1 ml of 40 mM-*p*-nitrophenyl phosphate as substrate. After incubation for 30 min, the reaction was stopped with 2.0 ml of 1 M-Tris/HCl/0.4 M-K₂HPO₄, pH 8.5, and the absorbance was read at 405 nm.

For both assays the reaction was linear with respect to the amount of enzyme and the time of incubation. A direct effect of the diphosphonates on the assays could be excluded, since the content of diphosphonates in cultured calvaria cells (Fast *et al.*, 1978) was such that the amount present in the reaction mixture was below 0.5 nmol. Amounts of diphosphonate 25 times higher than this value did not influence either enzyme activity.

Determination of K_m

Enzyme solution (0.1 ml; extracted with Triton X-100) was incubated at 37°C in 2 ml of buffer containing 10 mM-MgCl₂, 0.1 M-Na₂CO₃, pH 10.2, and different concentrations of *p*-nitrophenyl phosphate. The change in absorbance at 400 nm was monitored continuously on a recorder and the linear part of the curve was used to calculate the enzyme activity. Lineweaver-Burk plots were drawn and the K_m values were determined.

Heat inactivation

The same volumes of enzyme solutions (from control and dichloromethanediphosphonate-treated cells) with a protein concentration of 85 µg/ml (butanol-extracted) were incubated at 65°C or 56°C for 20 min. At time intervals of 5 min, samples were taken and the alkaline phosphatase activities were directly measured.

Polyacrylamide-gel electrophoresis

Electrophoresis of protein solutions was performed by the method of Davis (1964), except that the sample gel was not used. Enzyme solution, containing 10% (w/v) sucrose, was applied directly to the small-pore gel. Protein was stained with Amido Black (Davis, 1964) and alkaline phosphatase activity was stained by incubating the gel at room temperature in solution containing 2 mg of 2-naphthyl phosphate/ml, 1 mg of Fast Blue/ml, 1 mM-MgCl₂ and 0.06 M-boric acid adjusted with KOH to pH 9.7. The intensity of the bands of the alkaline phosphatase activity was determined by measuring the absorbance at 550 nm with a gel scanner (model 2410, Gilford, Oberlin, OH, U.S.A.).

Results

Dose response

Cells that had been cultured in the presence of 1-hydroxyethane-1,1-diphosphonate or dichloromethanediphosphonate possessed more alkaline phosphatase activity than cells cultured in the absence of diphosphonates (Table 1). Dichloromethanediphosphonate was much more effective than 1-hydroxyethane-1,1-diphosphonate, and increased the activity up to 30 times that of the control. This effect was dose-dependent and increased with time. In the medium no difference in enzyme activity was found between control and diphosphonate-treated cells. Acid phosphatase activity changed only slightly.

Time course

Fig. 1(a) shows the time course of increase in alkaline phosphatase caused by dichloromethanediphosphonate given on day 1. Enzyme activity was signi-

Table 1. Effect of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on the alkaline and acid phosphatase of cultured calvaria cells

The cells were cultured for 7 and 10 days. The diphosphonates were added on day 1. The mean $\pm$ S.E.M. for four dishes is given. Statistical significance was determined by Student's *t* test: * significantly different from control, $P < 0.05$; † significantly different from control, $P < 0.005$; ‡ significantly different from control, $P < 0.001$.

Alkaline phosphatase activity (nmol/min per 10 ⁶ cells)						
	Control	1-Hydroxyethane-1,1-diphosphonate		Dichloromethanediphosphonate		
		0.025 mM	0.25 mM	0.0025 mM	0.025 mM	0.25 mM
After 7 days	6.85 $\pm$ 0.58	6.65 $\pm$ 0.64	12.24 $\pm$ 1.42*	8.86 $\pm$ 0.66	13.94 $\pm$ 1.72*	79.54 $\pm$ 7.09‡
After 10 days	5.15 $\pm$ 0.41	7.16 $\pm$ 0.37*	16.44 $\pm$ 1.28‡	7.67 $\pm$ 0.26†	21.87 $\pm$ 0.99‡	171.16 $\pm$ 15.89‡
Acid phosphatase activity (nmol/min per 10 ⁶ cells)						
After 7 days	18.28 $\pm$ 0.63	17.12 $\pm$ 0.67	18.08 $\pm$ 0.86	20.02 $\pm$ 0.85	20.86 $\pm$ 1.06	20.38 $\pm$ 1.96
After 10 days	20.08 $\pm$ 1.52	24.58 $\pm$ 0.69*	26.84 $\pm$ 1.98*	23.35 $\pm$ 0.75	28.87 $\pm$ 1.71*	25.32 $\pm$ 4.30

ificantly higher than that of the control by day 3 and increased further until day 15. In the control cells the activity stayed at roughly the same level. Acid phosphatase activity (Fig. 1b) was not influenced by dichloromethanediphosphonate up to day 6, and then decreased slightly. Comparison with the growth curve (Fig. 1c) shows that the earliest increase in alkaline phosphatase activity in the cells treated with dichloromethanediphosphonate was observed when the cell number had already reached a plateau.

When added on day 7, after the cells had reached confluence, the diphosphonates also increased alkaline phosphatase activity, dichloromethanediphosphonate being much more potent than 1-hydroxyethane-1,1-diphosphonate (Fig. 2a). The earliest effect of dichloromethanediphosphonate was again observed 2 days after its addition. Acid phosphatase

activity was only slightly influenced by the diphosphonates (Fig. 2b), and cell number was decreased by dichloromethanediphosphonate as previously reported (Fig. 2c) (Fast *et al.*, 1978).

Effect of thymidine

In order to investigate whether the effect of the diphosphonates was due to the development of a new cell type, an inhibitor of cell division was added with the diphosphonates. As seen in Fig. 3, the result for dichloromethanediphosphonate-treated cells was similar to that in the absence of thymidine (compare

Fig. 1. Time course of the effect of dichloromethanediphosphonate given on day 1

(a) Alkaline phosphatase activity; (b) acid phosphatase activity; (c) cell number. Either 0.25 mM-dichloromethanediphosphonate (Δ) or nothing (control, $\circ$) was given from day 1 (arrow) up to day 15. The medium was changed on days 1, 4, 7, 10 and 13. The means $\pm$ S.E.M. for four dishes are given; S.E.M. values smaller than the symbols were not drawn. The alkaline and the acid phosphatase activity are expressed as nmol of *p*-nitrophenyl phosphate hydrolysed/min per 10^6 cells.

Fig. 2. Time course of the effect of diphosphonates given at confluence

(a) Alkaline phosphatase activity; (b) acid phosphatase activity; (c) cell number. The cells were cultured for 7 days in the absence of diphosphonates. On day 7 new medium with or without diphosphonate was added (arrow). Means $\pm$ S.E.M. for four dishes are given; S.E.M. values smaller than the symbols were not drawn. $\circ$, No diphosphonate; $\square$, 0.25 mM-1-hydroxyethane-1,1-diphosphonate; Δ , 0.25 mM-dichloromethanediphosphonate. The alkaline phosphatase and the acid phosphatase activity are expressed as nmol of *p*-nitrophenyl phosphate hydrolysed/min per 10^6 cells.

with Fig. 2), which indicates that the effect is independent of cell division and therefore does not appear to result from cell selection. 1-Hydroxyethane-1,1-diphosphonate had no effect in the thymidine-treated cells. The curve for acid phosphatase (Fig. 3b) was similar to that obtained in the absence of thymidine. The curve for cell number (Fig. 3c) shows that 2mM-thymidine had stopped cell growth, since the cell number in the control cells and in the cells treated with 1-hydroxyethane-1,1-diphosphonate did not increase as in Fig. 2, but slightly decreased in the thymidine-treated cultures. In the dichloromethane-

diphosphonate-treated cells, cell number again decreased.

The experiment presented in Fig. 1 was repeated in the presence and absence of thymidine. Dichloromethanediphosphonate was added on day 1 and 2mM-thymidine was added on day 2. For alkaline phosphatase, the same increase caused by dichloromethanediphosphonate was observed as in the absence of thymidine (result not shown). The growth curve again showed that cell division was stopped by thymidine, since the cell number decreased slightly in the cultures not treated with dichloromethanediphosphonate and decreased even more in the treated ones.

Fig. 3. Effect of diphosphonates in the presence of thymidine (a) Alkaline phosphatase activity; (b) acid phosphatase activity; (c) cell number. The experiment was done at the same time as the one presented in Fig. 2. After 7 days of culture in the absence of diphosphonates new medium with or without diphosphonates and containing 2mM-thymidine was added (arrow). Means $\pm$ S.E.M. for four dishes are given; S.E.M. values smaller than the symbols were not drawn. ○, No diphosphonate; □, 0.25 mM-1-hydroxyethane-1,1-diphosphonate; △, 0.25 mM-dichloromethanediphosphonate. The alkaline phosphatase and acid phosphatase activities are expressed as nmol of p-nitrophenyl phosphate hydrolysed/min per 10⁶ cells.

Effect of cycloheximide on alkaline phosphatase activity

To investigate whether the effect of diphosphonates on alkaline phosphatase activity involved new protein synthesis, an inhibitor of protein synthesis was tested. Cycloheximide inhibited the increase in alkaline phosphatase activity (Fig. 4). This was true whether cycloheximide was added at the same time as di-

Fig. 4. Effect of cycloheximide on the induction of alkaline phosphatase activity

The cells were grown in the absence of diphosphonates. On day 6, 0.25 mM-dichloromethanediphosphonate was added to one part of the dishes. Cycloheximide (1 μ g/ml) was given on days 6, 7 or 8. Means $\pm$ S.E.M. for four dishes are given; S.E.M. values smaller than the symbols were not drawn. ○, No diphosphonate, no cycloheximide; □, 0.25 mM-dichloromethanediphosphonate, no cycloheximide; ●, 0.25 mM-dichloromethanediphosphonate and from day 6 on cycloheximide; △, 0.25 mM-dichloromethanediphosphonate and from day 8 on cycloheximide.

chloromethanediphosphonate or later. Cycloheximide had no marked effect on the cell number and the alkaline phosphatase activity of the control (no diphosphonate), as observed in a separate experiment (results not shown). Table 2 shows that cycloheximide at the concentration used drastically inhibits protein synthesis.

Effects of diphosphonates on protein synthesis

As shown in Tables 2 and 3, the two diphosphonates, especially dichloromethanediphosphonate, inhibited protein synthesis. This effect seemed to be

Table 2. *Effect of cycloheximide on protein synthesis*

The cells had been cultured for 7 days in the presence or absence of 0.25 mM-dichloromethanediphosphonate. Then the cells were incubated for 24 h with medium containing 7.5 μ Ci of L-[4,5- 3 H]lysine/ml, with and without 0.25 mM-dichloromethanediphosphonate and no or 1 μ g of cycloheximide/ml. The radioactivity incorporated into the cells is given as the mean $\pm$ S.E.M. for four dishes except in the control, where only two dishes were used. * Significantly different from dichloromethanediphosphonate-treated cells, $P < 0.001$ (Student's t test).

Treatment	Radioactivity incorporated into 10^6 cells	
	(c.p.m.)	(%)
None	143970, 163030	100
Dichloromethane-diphosphonate	114427 $\pm$ 8251	74.5
Dichloromethane-diphosphonate + cycloheximide	16303 $\pm$ 1061*	10.6

greater when the diphosphonates were added on day 6 and protein synthesis was measured from day 8 to 9, than when the diphosphonates were added on day 1 and protein synthesis was measured from day 7 to 8, although in the latter case the exposure time to the diphosphonates was longer.

Effect of subcultivation

Cells treated with dichloromethanediphosphonate were subcultured in a medium free of diphosphonates. Alkaline phosphatase activity fell drastically, reaching a minimum within 2 days (Fig. 5). However, there was also a fall in enzyme activity in the control cells, so that the ratio in treated/control cells decreased more slowly, the values being 7.3 on day 7, 5.9 on day 8, 6.8 on day 9, 6.7 on day 10, 8.7 on day 11, 2.5 on day 13 and 3.2 on day 14. The ratio did not reach 1 during the experiment. If the treatment with dichloromethanediphosphonate was continued after subcultivation, the enzyme activity also decreased initially. However, the ratio of activity in treated to that in control cells did not, the values being 6.0 on day 8, 10.1 on day 9, 9.3 on day 10, 14.9 on day 11, 7.5 on day 13 and 10.9 on day 14.

Effect of parathyrin

Since it had been found that parathyrin inhibits the induction of alkaline phosphatase activity by cortisol or 5-iodo-2'-deoxyuridine in HeLa cells (Thomas & Goz, 1976), the effect of parathyrin on the increase produced by dichloromethanediphosphonate was tested. Parathyrin decreased the alkaline phosphatase activity both in the presence and absence of di-

Table 3. *Effect of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on the protein synthesis*

The cells were cultured in the presence or absence of 0.25 mM-1-hydroxyethane-1,1-diphosphonate or 0.25 mM-dichloromethanediphosphonate and incubated for 24 h with 7.5 μ g of L-[4,5- 3 H]lysine/ml of medium. The radioactivity incorporated into the cells is given as the mean $\pm$ S.E.M. for four dishes. Statistical significance was determined by Student's t test: * significantly different from control, $P < 0.02$; † significantly different from control, $P < 0.01$; ‡ significantly different from control, $P < 0.001$.

Treatment	Radioactivity incorporated into 10^6 cells	
	(c.p.m.)	(%)
Diphosphonates added for days 1-8, [3 H]lysine added from day 7 to 8		
No diphosphonate	131866 $\pm$ 4605	100
1-Hydroxyethane-1,1-diphosphonate	128003 $\pm$ 3986	97.1
Dichloromethanediphosphonate	107901 $\pm$ 4553†	81.8
Diphosphonates added for days 6-8, [3 H]lysine added from day 7 to 8		
No diphosphonate	142983 $\pm$ 7088	100
1-Hydroxyethane-1,1-diphosphonate	156552 $\pm$ 8340	109.5
Dichloromethanediphosphonate	135306 $\pm$ 15917	94.6
Diphosphonates added for days 6-9, [3 H]lysine added from day 8 to 9		
No diphosphonate	154095 $\pm$ 4517	100
1-Hydroxyethane-1,1-diphosphonate	132313 $\pm$ 5178*	85.8
Dichloromethanediphosphonate	79507 $\pm$ 4138‡	51.6
		1979

chloromethanediphosphonate (Table 4). However, the ratio of activity in control to that in treated cells did not change, except at the highest hormone concentration where induction was decreased.

Gel electrophoresis

Alkaline phosphatase activity in control cells migrated as one strong band with an R_F value of 0.14. The enzyme activity from dichloromethanediphos-

phonate-treated cells also migrated as one strong band with the same R_F value of 0.14, but a minor band with an R_F value of 0.25 was also observed. The absorbance of this minor band was about 7% of that of the strong band. The same amount of enzyme

Fig. 5. Effect of subcultivation

Cells were grown from day 1 to 7 in the presence or absence of 0.25 mM-dichloromethanediphosphonate. On day 7 (arrow), the cells were released from the dish by digestion with collagenase and trypsin, and were subcultured. Alkaline phosphatase activity is given as mean $\pm$ S.E.M. for four dishes; S.E.M. values smaller than the symbols were not drawn. ○, No diphosphonates; Δ, 0.25 mM-dichloromethanediphosphonate from day 1 to 14; ▲, 0.25 mM-dichloromethanediphosphonate from day 1 to 7.

Fig. 6. Heat inactivation of alkaline phosphatase activity

The experimental procedure is described in the Methods section. Alkaline phosphatase activity was extracted with butan-1-ol from untreated cells (specific activity 0.37 μ mol of p-nitrophenyl phosphate/min per mg of protein) (●) or from cells treated from day 1 to 7 with 0.25 mM-dichloromethanediphosphonate (specific activity 2.23 μ mol of p-nitrophenyl phosphate/min per mg of protein) (□). The enzyme activity is given as percentage of the control value.

Table 4. Effect of parathyrin on the induction of alkaline phosphatase

The cells were grown in the presence or absence of 0.25 mM-dichloromethanediphosphonate from day 1 to 4. Bovine parathyrin solution (5 μ l containing 2 mg of bovine serum albumin/ml, 0.15 M-NaCl, 0.01 M-HCl and 1.0 mM-mercaptoethanol) or the same solution, but containing no parathyrin (5 μ l) was added daily from day 1 to 3 to increase the parathyrin concentration by 0.2, 1.0 or 5.0 units/ml. The alkaline phosphatase activity of the cells was determined on day 4 and is given as the mean $\pm$ S.E.M. for four dishes. Statistical significance was determined by Student's *t* test: * significantly different from control, $P < 0.001$; † significantly different from control, $P < 0.05$.

Parathyrin concentration (units/ml)	Alkaline phosphatase activity (nmol/min per 10 ⁶ cells)		
	Control (C)	Dichloromethane-diphosphonate (DP)	$\frac{DP}{C} \times 100$ (%)
0	33.3 $\pm$ 1.7	73.8 $\pm$ 3.8*	222
0.2	23.5 $\pm$ 1.7	59.8 $\pm$ 4.6*	254
1.0	11.0 $\pm$ 1.1	28.2 $\pm$ 2.1*	256
5.0	6.7 $\pm$ 0.6	9.1 $\pm$ 0.6†	136

activity was put on the gel in each case, so it was unlikely that the minor band was not detected in the case of the control enzyme.

Heat inactivation

Alkaline phosphatase activity of control cells and of cells treated with dichloromethanediphosphonate decreased at the same rate when heated at 56°C (Fig. 6). At 65°C both activities were inactivated completely in less than 5 min (results not shown).

Effects of inhibitors

(-)-*p*-Bromotetramisole, a potent inhibitor of alkaline phosphatase (Borgers & Thoné, 1975), (+)-*p*-bromotetramisole, which is 100 times less potent, and L-phenylalanine all inhibited alkaline phosphatase activity in the control cells and the dichloromethanediphosphonate-treated cells to the same extent (Figs. 7 and 8).

Michaelis constants

The K_m of the alkaline phosphatase activity was 2.78 ± 0.43 mM (3) [mean $\pm$ S.E.M. (n)] for the control cells and 3.09 ± 0.24 mM (3) for the cells treated with

Fig. 7. Inhibition of alkaline phosphatase activity by *p*-bromotetramisole

Alkaline phosphatase activity was extracted with Triton X-100 from untreated cells (specific activity $0.099 \mu\text{mol}$ of *p*-nitrophenyl phosphate/min per mg of protein; ○, ●) or from cells treated with 0.25 mM-dichloromethanediphosphonate from day 1 to 7 (specific activity $0.48 \mu\text{mol}$ of *p*-nitrophenyl phosphate/min per mg of protein; □, ■). The inhibition by (-)-*p*-bromotetramisole (○, □) and (+)-*p*-bromotetramisole (●, ■) was measured. The enzyme activity (mean of duplicates) is given as percentage of the control value in the absence of *p*-bromotetramisole.

dichloromethanediphosphonate from day 1 to 7. The two values are not significantly different.

Discussion

Diphosphonates, especially dichloromethanediphosphonate, were found to increase alkaline phosphatase activity in cultured calvaria cells. On the other hand, no effects were found in cultured cartilage cells (H. Guenther & H. Fleisch, unpublished work). Since 2 mM-thymidine, an inhibitor of cell growth, had no effect on this stimulation, the latter is independent of cell growth. The physical and chemical characteristics of the two enzyme activities were similar. It seems therefore that the increased enzyme activity originates from the same protein as that from the control cells.

The stimulation of the alkaline phosphatase activity could be inhibited by cycloheximide, an inhibitor of protein synthesis. This has also been observed in other systems (Griffin & Cox, 1966; Koyama *et al.*, 1972; Chou & Robinson, 1977b; Melnykovich & Lopez, 1977; Deutsch *et al.*, 1977). However, it does not necessarily mean that the effect is due to synthesis of new enzyme. Indeed, Cox *et al.* (1971) have found by immunological methods that the increase in enzyme activity induced in HeLa cells by glucocorticoids is not due to increased quantities of enzyme protein, but may result from the synthesis of modifier molecule.

Bazzell *et al.* (1976) have found that the HeLa-cell alkaline phosphatase is a phosphoprotein and that the

Fig. 8. Inhibition of alkaline phosphatase activity by L-phenylalanine

The same enzyme preparation as in Fig. 7 was used. The enzyme activity (mean $\pm$ S.E.M.) ($n = 4$) is given as a percentage of the control value in the absence of L-phenylalanine. ○, Enzyme activity extracted from untreated cells; □, enzyme activity extracted from cells treated with 0.25 mM-dichloromethanediphosphonate.

induced form of the enzyme has only one-half of the phosphate residues associated with the control enzyme. Thus, by inhibiting some protein kinases, the diphosphonates might generate an alkaline phosphatase with fewer phosphate residues.

Most of the compounds that increase the alkaline phosphatase of cultured cells show some inhibition of the growth of the cells (Cox & MacLeod, 1961; Koyama & Ono, 1972; Speeg *et al.*, 1977; Chou & Robinson, 1977a,b; Deutsch *et al.*, 1977). This is also true for dichloromethanediphosphonate, as seen in this and in earlier work (Fast *et al.*, 1978).

Dichloromethanediphosphonate does not seem to have a mutagenic effect, since after subcultivation in a medium free of diphosphonates the activity of the alkaline phosphatase stayed low. The reason why it did not revert completely to control values may be that there was a slow release of diphosphonates from the cell. It is known that the uptake of the diphosphonates is slow (Fast *et al.*, 1978). A decrease in enzyme activity after culture in a medium free of inducer has also been observed by others (Griffin & Cox, 1966; Koyama & Ono, 1972; Chou & Robinson, 1977b).

It is known that alkaline phosphatase can be increased in HeLa cells by substrates (Cox & Pontecorvo, 1961). Since PP_i is a substrate of alkaline phosphatase (Fernley & Bisaz, 1968) and since the diphosphonates are analogues of PP_i , containing a P-C-P instead of a P-O-P bond, they might stimulate like a substrate. In contrast with PP_i , diphosphonates are not enzymically hydrolysed and therefore they might be more effective than PP_i . It has been shown that diphosphonates do interact with extracted alkaline phosphatase, stimulating or inhibiting the enzyme activity (Wöltgens *et al.*, 1973; Felix & Fleisch, 1974, 1976; Kelly *et al.*, 1974).

The effect on alkaline phosphatase is not restricted to conditions *in vitro*, but can also be obtained *in vivo*. Thus, in the epiphyseal growth plate, both the total alkaline phosphatase activity and that contained in the matrix vesicles are increased when chickens are treated with diphosphonates (Felix *et al.*, 1978). Therefore there is some correlation between the effect on cultured cells and the effect *in vivo*, although *in vivo* 1-hydroxyethane-1,1-diphosphonate was more effective than dichloromethanediphosphonate.

Whether this effect on phosphatase has any correlation with the effects of diphosphonates on calcium metabolism *in vivo* is so far only an intriguing speculation.

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