The effects of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on collagen synthesis by rabbit articular chondrocytes and rat bone cells

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Investigations were performed to assess the effects of dichloromethanediphosphonate on the synthesis of collagen by (1) isolated rabbit articular chondrocytes, (2) isolated rat calvaria bone cells and (3) bone explants from rats treated with the diphosphonates. The studies showed that dichloromethanediphosphonate, but not 1-hydroxyethane-1,1-diphosphonate, causes articular chondrocytes to increase net collagen biosynthesis, both when measured as ^3H -labelled or as non-radioactive material, in a dose-related fashion. The increment in collagen synthesis was still evident with cells that were exposed continuously to the diphosphonate in primary as well as secondary culture; however, it declined with cells in tertiary culture and was absent after the fourth subculture. The type of collagen was not affected by the diphosphonate. The synthesis of collagen by bone cells was likewise increased with dichloromethanediphosphonate. No effects were detected when 1-hydroxyethane-1,1-diphosphonate was tested. Finally, when calvaria and tibiae from diphosphonate-treated rats were cultured *in vitro*, the positive effect of dichloromethanediphosphonate on collagen synthesis was also evident. 1-Hydroxyethane-1,1-diphosphonate, on the other hand, decreased the incorporation of [^3H]proline into the collagen of calvaria and osseous tibial shafts and showed no effect on the collagen synthesis of the cartilaginous tibial heads.

The diphosphonates are a group of synthetic phosphates characterized by a P–C–P bond and therefore structurally related to PP_i (inorganic pyrophosphate), which contains a P–O–P bond. Unlike PP_i , the diphosphonates are resistant to hydrolysis by endogenous phosphatase to yield P_i (inorganic phosphate).

Of the various diphosphonates studied, 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate have been shown to prevent ectopic calcification *in vivo* (Fleisch *et al.*, 1970) and evoke profound alterations on skeletal tissues. Both compounds affect the formation (Fleisch *et al.*, 1968, 1970; Francis, 1969; Francis *et al.*, 1969) and the dissolution (Fleisch *et al.*, 1969; Russell *et al.*, 1970) of hydroxyapatite *in vitro*. *In vivo*, high doses of 1-hydroxyethane-1,1-diphosphonate, but not dichloromethanediphosphonate, inhibit the accretion of mineral into bone (Jowsey *et al.*, 1970; King *et al.*, 1971; Schenk *et al.*, 1973), whereas dichloromethanediphosphonate was found to be more potent than 1-hydroxyethane-1,1-diphosphonate in preventing bone resorption *in vivo* (Gasser *et al.*, 1972; Schenk *et al.*, 1973) as well as *in vitro*

(Fleisch *et al.*, 1969; Reynolds *et al.*, 1972; Russell *et al.*, 1970). Whereas formerly these effects had been attributed mainly to chemisorption of diphosphonates to calcium phosphate mineral surfaces, exerting a retardatory action *in vitro* on mineral-ion efflux and influx from mineral surfaces (Francis *et al.*, 1973; Fleisch *et al.*, 1973), increasing data have now become available showing that diphosphonates also influence metabolic events in various cell types (Fast *et al.*, 1978; Felix & Fleisch, 1979; Felix *et al.*, 1980; Guenther *et al.*, 1979) and suggesting that changes in skeletal tissues caused by the diphosphonates may be due, at least in part, to cell-mediated mechanisms (Fleisch & Felix, 1979). Whereas these investigations were directed towards problems pertaining to intracellular metabolic events, the present work was undertaken to examine the effects of diphosphonates on extracellular matrix formation. We showed previously (Guenther *et al.*, 1979) that articular chondrocytes cultured in the presence of dichloromethanediphosphonate synthesize increased amounts of proteoglycans. The purpose of the present investigation was to determine whether chondrocytes and also

bone cells when cultured in the presence of diphosphonates will also increase the synthesis of collagen. Furthermore, an attempt was made to examine the effects *in vivo* of dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate on the biosynthesis of collagen.

The results show that dichloromethanediphosphonate, but not 1-hydroxyethane-1,1-diphosphonate, stimulates collagen biosynthesis of chondrocytes and also bone cells, both when added *in vitro* and administered *in vivo* to young rats.

Experimental

Materials

Eagle's minimum essential medium (Eagle, 1959), Ham's F-12 medium (Ham, 1965), foetal-calf serum and penicillin/streptomycin/fungizone solution were all purchased from Seromed, Fakola A.G., Basel, Switzerland. Collagenase (type CLS II) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Trypsin, pepsin and β -aminopropionitrile fumarate were supplied by Sigma Chemical Co., St. Louis, MO, U.S.A. L-[2,3- ^3H]-Proline (20–40 Ci/mmol) was obtained from New England Nuclear Corp., Dreieichenhain, Germany. Reagents for the polyacrylamide-gel electrophoresis were purchased from Bio-Rad Laboratories I.G., Zurich, Switzerland. Triton X-100 and butyl-PBD[5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole used in liquid-scintillation counting were purchased from Siegfried A.G., Zofingen, Switzerland and CIBA-Geigy, Basel, Switzerland, respectively. 1-Hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate were gifts from Procter and Gamble Co., Cincinnati, OH, U.S.A. All other chemicals and reagents were of analytical grade and purchased from E. Merck A.G., Darmstadt, Germany.

Methods

Isolation and culture of rat calvaria cells and rabbit articular chondrocytes. (a) Calvaria cells. Isolation of rat calvaria bone cells was performed as described by Fast *et al.* (1978).

(b) Articular chondrocytes. The preparation and subsequent cultivation of rabbit articular chondrocytes were performed essentially as outlined by Guenther *et al.* (1979).

Culturing the cells. Both cell types were cultured in a similar fashion, with the exception that calvaria bone cells were cultivated in minimum essential medium with Earle's balanced salt solution, and articular chondrocytes were cultivated in Ham's F-12 medium. After cell attachment, which occurred by 24 h, fresh media were added that contained either dichloromethanediphosphonate or 1-hydroxyethane-1,1-diphosphonate.

Preparation of chondrocyte subcultures. Chon-

drocyte subcultures were prepared as described by Guenther *et al.* (1979).

Measurement of collagen biosynthesis in cell culture. In experiments designed to study the biosynthesis of collagen in the presence of dichloromethanediphosphonate or 1-hydroxyethane-1,1-diphosphonate, newly confluent bone or cartilage cells were incubated for 24 h in minimum essential medium and Ham's F-12 medium respectively containing 10% (v/v) foetal-calf serum, either 1-hydroxyethane-1,1-diphosphonate or dichloromethanediphosphonate in concentrations of 250 mM or 25 mM, 100 μg of ascorbic acid/ml, 100 μg of β -aminopropionitrile fumarate/ml, 10 μCi of L-[2,3- ^3H]proline/ml and 1% penicillin/streptomycin solution. The incubation was performed under CO_2 /air (1:19) at 37°C.

To determine the distribution of newly synthesized labelled collagen deposited into the cell layer (pericellular space) and secreted into the growth medium, the latter was carefully removed. The cell layer was rinsed three times with 2 ml of ice-cold 0.9% NaCl and the wash was pooled with the medium. To each of the culture media, 1 mg of purified acid-soluble rat skin collagen/ml (Strawich & Nimni, 1971) was added as carrier, after which the media were dialysed against 0.5 M-acetic acid at 4°C for 72 h. The acetic acid was changed twice every day. An additional dialysis step of 48 h against cold deionized water resulted in a precipitation of the collagen, which was collected by centrifugation at 10000 *g* for 30 min. The collagen pellet was then redissolved in 0.5 M-acetic acid and the resulting solution was centrifuged at 44000 *g* for 1 h to remove material that had not redissolved. The pellet thus obtained was discarded. The clear collagen solution was used to analyse the amount of radioactivity that had become associated with newly synthesized collagen secreted into the growth medium.

To determine the quantity of collagen synthesized and deposited into the cell layer, the collagen was extracted from the latter by adding 3 ml of cold 0.5 M-acetic acid containing pepsin (1 mg/ml) to each culture dish. The extraction was performed by gentle shaking on a metabolic shaker at 4°C for 24 h. Before the addition of acetic acid/pepsin, acid-soluble rat skin collagen was added at a concentration of 1 mg/ml to act as carrier. After termination of the pepsin extraction, the samples were freeze-dried, then redissolved in 3 ml of 1 M-NaCl/0.05 M-Tris buffer, pH 8.6, and finally dialysed against the same buffer at 4°C for 72 h. Material that did not dissolve, or appeared after dialysis, was removed by centrifugation at 22000 *g* for 30 min. The supernatant was used to determine the radioactivity associated with intact collagen.

Organ culture. (a) Preparation of animals. Wistar

rats (1 day old, and bred in our Institute) were injected subcutaneously daily for 10 days with 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate at a dose of 10 and 1 mg of P/kg body weight. Both diphosphonates were dissolved in 0.9% NaCl and adjusted to pH 7.3. Control animals received equivalent volumes of saline (0.9% NaCl). Body weights of the animals were determined every day before the injections. After completion of the treatment the young rats were killed by decapitation. Thereafter calvaria and tibiae were excised and immediately placed in cold minimum essential medium with Earle's salts (Eagle, 1959) until all explants were dissected.

(b) Culture of calvaria and tibia. The explants were then rinsed twice with minimum essential medium and transferred to culture dishes [10 explants/dish (60 mm diam.)] to which 4 ml of fresh medium was added containing 30 μ Ci of [3 H]proline/ml, 100 μ g of ascorbic acid/ml, 100 μ g of β -aminopropionitrile fumarate/ml, 10% (v/v) foetal-calf serum and 1% penicillin/streptomycin solution, but no diphosphonates. After incubation for 24 h at 37°C under CO₂/air (1:19), the explants were taken from the culture media and rinsed three times with cold deionized water containing 1 mg of non-radioactive proline/ml. Growth media were not processed and therefore discarded.

Isolation of [3 H]collagen from cultured explants. Calvaria and tibiae were cleaned from fascia, adherent cells and cell debris. Bone marrow trapped in the tibial shafts was removed effectively with the aid of a syringe equipped with a fine injection needle after the tibial heads had been separated from the shafts. These tibial heads, which proved to consist almost entirely of cartilaginous tissue, were processed as separate tissues and used to assess the effect(s) of 1-hydroxyethane-1,1-diphosphonate or dichloromethanediphosphonate on the biosynthesis of cartilage collagen. After the tissue specimens were pulverized with a bone mill by using liquid N₂, they were subjected to demineralization with 0.5 M-EDTA, pH 7.9, at 4°C for 7 days. Resulting demineralized tissue samples were then extracted with pepsin/acetic acid (1 mg of pepsin/1 ml of 0.5 M-acetic acid) for 48 h at 4°C with gentle shaking. Resulting extracts were clarified by centrifugation at 22 000 g for 45 min and subsequently adjusted to pH 8.6 with 1 M-NaOH. The extracts were then extensively dialysed against 1 M-NaCl/0.5 M-Tris buffer, pH 8.6. The pepsin-insoluble residues were discarded after it was established that less than 1% of total radioactivity incorporated into collagen remained unextracted.

Determination of the chain composition of collagen. The identification of the chain composition of bone (type I) and cartilage (type II) collagen was achieved by dodecyl sulphate/5% (w/v) polyacryl-

amide-gel electrophoresis (Benya *et al.*, 1977). The gels were sliced into 1 mm discs and placed into scintillation vials to which 0.5 ml of a 3% (v/v) H₂O₂ solution was added. The vials were then transferred into a drying oven and were kept there for 2 h at 60°C and subsequently counted for radioactivity in a liquid-scintillation counter.

Chemical and biochemical methods. (a) Hydrolysis of collagen for the determination of hydroxyproline was performed with 6 M-HCl at 108°C for 18 h in sealed tubes that were extensively flushed with N₂. HCl was removed by evaporation. The occasional colour that developed in the hydrolysed samples was removed with charcoal.

(b) DNA was prepared and determined as described by Hinegardner (1971).

(c) The colorimetric determination of hydroxyproline was as described by Stegemann & Stalder (1967), a Technicon automatic analyser being used.

(d) The radioactivity was measured routinely in triplicates with a scintillation fluid consisting of 750 ml of toluene, 250 ml of Triton X-100, 7 g of butyl-PBD and 80 g of naphthalene.

If not otherwise stated, all results given in the present paper are for one representative experiment from a minimum of three experiments.

Results

Effect of diphosphonates on the incorporation of [3 H]proline into collagen synthesized by rabbit articular chondrocytes

Table 1 provides evidence that chondrocytes treated with dichloromethanediphosphonate apparently also synthesize elevated amounts of collagen, as shown by the increase in [3 H]proline incorporation into newly synthesized collagen. The increments in labelled collagen proved to be dose-related and were determined to amount to approx. 20 and 50% for collagen associated with the cell layer and 32 and 70% for collagen found in the growth medium of cells exposed to 25 μ M- and 250 μ M-dichloromethanediphosphonate respectively. 1-Hydroxyethane-1,1-diphosphonate did not increase [3 H]proline incorporation into collagen.

Effect of diphosphonates on the synthesis of collagen by chondrocytes as determined by the content of hydroxyproline in the growth medium

To ascertain whether dichloromethanediphosphonate truly stimulates chondrocytes to increase collagen synthesis or whether the effects observed may possibly be due to an radioisotopic artefact, hydroxyproline determinations were performed to determine the actual amount of collagen secreted into the growth media. The data listed in Table 2, confirmed by two independent experiments, show that the increase in [3 H]proline incorporation in-

Table 1. *Incorporation of [³H]proline into collagen synthesized by rabbit articular chondrocytes in culture in the absence or presence of diphosphonates*

Chondrocytes grown to confluence with or without diphosphonates were pulsed with 10 μ Ci of L-[2,3-³H]proline/ml for 24 h. Collagen was prepared as described in the Experimental section. Radioactivity incorporated into newly synthesized collagen is given in d.p.m./ μ g of DNA. Results are expressed as means $\pm$ S.E.M., the number of dishes being given in parentheses.

Addition	Concn.	Radioactivity incorporated (d.p.m./ μ g of DNA)	
		Cell layer	Medium
Control		19 750 $\pm$ 620 (5)	23 370 $\pm$ 340 (5)
1-Hydroxyethane-1,1-diphosphonate	250 μ M	19 160 $\pm$ 550 (5)*	23 100 $\pm$ 430 (5)*
Dichloromethane-diphosphonate	25 μ M	23 750 $\pm$ 530 (5)†	30 900 $\pm$ 1100 (5)†
Dichloromethane-diphosphonate	250 μ M	30 470 $\pm$ 650 (5)†	39 930 $\pm$ 2530 (5)†

* Statistically not significant.

† $P < 0.001$ (control versus dichloromethanediphosphonate-treated).

Table 2. *Biosynthesis of collagen by rabbit articular chondrocytes in the absence or presence of diphosphonates*

Chondrocytes were grown in the presence or absence of either diphosphonate. Collagen secreted by the cells into the growth media was measured by hydroxyproline content of hydrolysed collagen. To obtain collagen concentration, hydroxyproline values were multiplied by a factor of 7.41. Results are expressed as means $\pm$ S.E.M., the number of dishes being given in parentheses.

Addition	Concn.	Collagen (μ g/ μ g of DNA)	
		Expt. I	Expt. II
Control		1.12 $\pm$ 0.08 (3)	0.65 $\pm$ 0.03 (5)
1-Hydroxyethane-1,1-diphosphonate	250 μ M	1.00 $\pm$ 0.03 (3)*	—
Dichloromethane-diphosphonate	250 μ M	1.59 $\pm$ 0.12 (3)†	0.88 $\pm$ 0.03 (5)‡
Dichloromethane-diphosphonate	25 μ M	—	0.69 $\pm$ 0.05 (5)*

* Statistically not significant.

† $P < 0.025$ (control versus dichloromethanediphosphonate-treated).

‡ $P < 0.005$ (control versus dichloromethanediphosphonate-treated).

duced by dichloromethanediphosphonate constituted a real stimulation of collagen synthesis. Furthermore, 1-hydroxyethane-1,1-diphosphonate appears to be biologically inactive with respect to collagen synthesis.

Effect of dichloromethanediphosphonate on collagen synthesized by chondrocytes plated in high density

Dichloromethanediphosphonate, when tested in concentrations where it was found to alter collagen synthesis, induced a concomitant moderate decrease in total cell number per dish (Guenther *et al.*, 1979). To eliminate the possibility that the increment in collagen production was partly or perhaps entirely due to a decrease in cell density, chondrocytes were plated at three times their usual number.

A similar large enhancement of [³H]proline incorporation into cell-layer collagen and collagen found in the growth medium was evident, although the DNA content per dish was not affected by the diphosphonate (results not shown).

Short-term effect of dichloromethanediphosphonate on collagen synthesis by chondrocytes

In the experiments described above, chondrocytes were cultured to confluence in the absence of dichloromethanediphosphonate. To determine the short-term effect of the diphosphonate, [³H]proline, along with dichloromethanediphosphonate, was added to the cell cultures, and after 24 h the synthesis of collagen was measured. The data obtained revealed that the diphosphonate failed to

evoke a change in the synthesis of collagen by the cells within this 24 h time span (results not shown).

Long-term effect of diphosphonates on the synthesis of collagen by chondrocytes grown in subcultures

To test the long-term effect of dichloromethane-diphosphonate and 1-hydroxyethane-1,1-diphosphonate on collagen synthesis, cells were passaged and subcultured four consecutive times, the diphosphonates being present at all times. The results of these experiments are summarized in Table 3. The Table shows that chondrocytes grown with dichloromethanediphosphonate in first and second sub-

culture respond equally; however, after the third passage the stimulatory effect was already considerably decreased and reached a value of merely 14% above control after the fourth passage. In primary cultures, 1-hydroxyethane-1,1-diphosphonate did not exert any effect on the cells, but once the cells were grown in subculture, this diphosphonate displayed an inhibitory action towards collagen synthesis. It is noteworthy that chondrocytes after the fourth passage incorporate considerably more radioactivity into collagen than in the preceding subcultures. This phenomenon occurs regardless of the diphosphonate treatment.

Table 3. *Long-term effect of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on the incorporation of [^3H]proline into collagen synthesized by articular chondrocytes*

Chondrocytes were grown in primary culture to confluence in the absence or presence of 250 μM -1-hydroxyethane-1,1-diphosphonate or 250 μM -dichloromethanediphosphonate. One portion of the cells were pulsed with 10 μCi of [^3H]proline for 24 h; the other portion was used to pass the cells three more times. The results represent total radioactivity incorporated into collagen recovered from cell layer and medium and are expressed as means $\pm$ S.E.M., the number of dishes being given in parentheses.

Passage		Incorporation of radioactivity (d.p.m./ μg of DNA)	Treated Control
First	Control	23 080 $\pm$ 460 (5)	—
	1-Hydroxyethane-1,1-diphosphonate	17 450 $\pm$ 570 (5)*	0.76
	Dichloromethanediphosphonate	34 890 $\pm$ 1030 (5)†	1.51
Second	Control	34 460 $\pm$ 1130 (5)	—
	1-Hydroxyethane-1,1-diphosphonate	33 100 $\pm$ 1500 (5)	0.96
	Dichloromethanediphosphonate	53 010 $\pm$ 1380 (4)†	1.54
Third	Control	47 300 $\pm$ 640 (5)	—
	1-Hydroxyethane-1,1-diphosphonate	35 900 $\pm$ 500 (5)*	0.76
	Dichloromethanediphosphonate	63 490 $\pm$ 1400 (4)†	1.33
Fourth	Control	168 200 $\pm$ 4500 (5)	—
	1-Hydroxyethane-1,1-diphosphonate	131 800 $\pm$ 1400 (5)*	0.78
	Dichloromethanediphosphonate	191 500 $\pm$ 4125 (4)†	1.14

* $P < 0.001$ (control versus 1-hydroxyethane-1,1-diphosphonate-treated).

† $P < 0.001$ (control versus dichloromethanediphosphonate-treated).

Table 4. *Effect of diphosphonates on the α -polypeptide chain composition of collagen synthesized by rabbit articular chondrocytes*

[^3H]Proline-labelled collagen synthesized by chondrocytes in primary and tertiary culture in the absence and presence of 250 μM -1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate was separated into its α -polypeptide chain by dodecyl sulphate/polyacrylamide-gel electrophoresis. The data in Table were obtained from a pool of five separate dishes and represent radioactivity associated with the individual α -chains expressed as percentage of the total radioactivity recovered from the polyacrylamide gels.

		Radioactivity (% of total)					
		Primary culture			Tertiary culture		
Chain(s)	...	$\alpha 1$	$\alpha 2$	Ratio $\alpha 1/\alpha 2$	$\alpha 1$	$\alpha 2$	Ratio $\alpha 1/\alpha 2$
Control		83.3	—	—	59.3	26.5	2.24
1-Hydroxyethane-1,1-diphosphonate		83.4	—	—	66.7	22.4	2.98
Dichloromethanediphosphonate		84.6	—	—	63.0	26.4	2.39

Effect of diphosphonates on the type(s) of collagen synthesized by chondrocytes in primary and third subculture

Mayne *et al.* (1976) and others have shown that the chondrocyte phenotype is unstable *in vitro*; after having gone through successive subcultures, they synthesize, apart from cartilage, specific type-II collagen {chain composition $[\alpha 1(\text{II})]_3$ } and also type-I collagen {chain composition $[\alpha 1(\text{I})]_2\alpha 2$ }. Table 4 presents the results of an experiment where the influence of diphosphonates on the chondrocyte phenotype was examined. Neither dichloromethane-diphosphonate nor 1-hydroxyethane-1,1-diphosphonate influenced the stability of the phenotype in primary culture; only chondrocyte specific type-II collagen was found to have been synthesized by these cultures.

Effect of diphosphonates on bone cell growth

As an index for cell growth, DNA determinations were performed on cells that reached monolayer with or without 250 μM -dichloromethanediphosphonate or 250 μM -1-hydroxyethane-1,1-diphosphonate.

Table 5. *Effect of diphosphonates on growth of rat calvaria bone cells*

After control cells had reached confluence, DNA determinations were performed on treated and non-treated cultures. Results are expressed as means $\pm$ s.e.m., the number of dishes being given in parentheses.

	DNA ($\mu\text{g}/\text{dish}$)	Growth inhibition (%)
Control	60.52 $\pm$ 3.75 (5)	—
1-Hydroxyethane-1,1-diphosphonate	52.19 $\pm$ 1.50 (5)*	13.76
Dichloromethane-diphosphonate	29.95 $\pm$ 0.95 (5)	50.51

* Statistically not significant.

phonate. The data, expressed as DNA/dish (Table 5), indicate that dichloromethanediphosphonate had caused a diminution of viable cells to approx. 50% as compared with controls. The effect of 1-hydroxyethane-1,1-diphosphonate on the number of cells per culture dish was considerably less severe; only a 14% decrease in cell population was noticed, which, on the basis of Student's *t* test, failed to reach statistical significance.

Effect of diphosphonates on the incorporation of [^3H]proline into collagen synthesized by rat calvaria bone cells

The results presented in Table 6 indicate that bone cells likewise are induced by dichloromethanediphosphonate to synthesize increased amounts of collagen, when grown in its presence. As shown above with chondrocytes, bone cells also did not respond to 1-hydroxyethane-1,1-diphosphonate. It may be worthwhile to mention that the increase in radioactively labelled collagen was measured only with collagen found in the growth medium. Surprisingly, no increase in [^3H]proline-labelled collagen was detected in the cell layer.

The incorporation in vitro of [^3H]proline into collagen of bone explants from rats treated in vivo with diphosphonates

The question of whether the effects *in vitro* of the diphosphonates on the synthesis of collagen by cartilage and bone cells would prevail when the compound is also administered *in vivo* is answered in Table 7. The incorporation *in vitro* of [^3H]proline into newly synthesized bone and cartilage collagen was increased by a substantial margin in tissues derived from young rats that had been treated with dichloromethanediphosphonate. Whereas the effects *in vivo* as well as *in vitro* of dichloromethanediphosphonate on collagen synthesis exhibit some degree of consistency, the results *in vivo* obtained with 1-hydroxyethane-1,1-diphosphonate deviated

Table 6. *Biosynthesis of collagen by rat calvaria bone cells in the absence or presence of diphosphonates*

Bone cells grown to confluence under the influence of 250 μM -1-hydroxyethane-1,1-diphosphonate or 250 μM -dichloromethanediphosphonate were pulsed with 10 μCi of L-[2,3- ^3H]proline/ml for 24 h. Collagen was prepared and isolated as described in the Experimental section. Radioactivity incorporated into newly synthesized collagen is given in d.p.m./ μg of DNA. Results are expressed as means $\pm$ s.e.m., the number of dishes being given in parentheses.

	Radioactivity incorporated (d.p.m./ μg of DNA)	
	Cell layer	Medium
Control	13 120 $\pm$ 560 (5)	29 370 $\pm$ 1090 (5)
1-Hydroxyethane-1,1-diphosphonate	15 312 $\pm$ 1190 (5)*	29 060 $\pm$ 1400 (5)*
Dichloromethanediphosphonate	14 060 $\pm$ 460 (5)*	82 500 $\pm$ 3440 (5)†

* Statistically not significant.

† $P < 0.001$ (control versus dichloromethanediphosphonate-treated).

Table 7. *Effect of diphosphonates on the incorporation of [³H]proline in vitro into collagen of bone explants from young rats that were treated in vivo with diphosphonates for 10 consecutive days*

Treatment of rats, cultivation of explants and treatment of tissues were performed as described in the Experimental section. Results represent total radioactivity incorporated/g of organic matrix (organic matrix means demineralized bone and cartilage after extraction with guanidinium chloride) obtained from separate experiments (with eight) to ten animals/expt.) and are expressed as means $\pm$ S.E.M., with the number of experiments in parentheses.

Tissue	Addition	Incorporation of radioactivity (d.p.m./mg of organic matrix)	Treated Control
Calvaria	Control	31 280 $\pm$ 2160 (3)	—
	1-Hydroxyethane-1,1-diphosphonate	18 620 $\pm$ 1600 (3)	0.59
	Dichloromethanediphosphonate	73 310 $\pm$ 5720 (3)	2.32
Tibia	Control	11 150 $\pm$ 920 (3) [§]	—
	1-Hydroxyethane-1,1-diphosphonate	8180 $\pm$ 630 (3) [*]	0.73
	Dichloromethanediphosphonate	16 530 $\pm$ 1710 (3) [†]	1.48
Cartilage	Control	4500 $\pm$ 720 (3)	—
	1-Hydroxyethane-1,1-diphosphonate	5440 $\pm$ 310 (3) [*]	1.20
	Dichloromethanediphosphonate	35 560 $\pm$ 5260 (3) [‡]	7.90

* Statistically not significant.

† $P < 0.005$ (control versus dichloromethanediphosphonate-treated).

‡ $P < 0.001$ (control versus dichloromethanediphosphonate-treated).

§ $P < 0.01$ (control versus 1-hydroxyethane-1,1-diphosphonate-treated).

|| $P < 0.001$ (control versus 1-hydroxyethane-1,1-diphosphonate-treated).

Table 8. *α -Chain composition of collagen synthesized by bone and cartilage tissues of rats treated with diphosphonates*

Treatment of rats, incubation of explants and determination of α -chains are described in the Experimental section. Values in the Table represent [³H]proline-labelled α -chain radioactivity expressed as a percentage of total radioactivity recovered from polyacrylamide gels.

³ H]Proline-labelled α -chain radioactivity (% of total)										
Chain(s) ...	Calvaria			Tibial shafts			Cartilage (tibial heads)			Ratio $\alpha 1/\alpha 2$
	$\alpha 1$	$\alpha 2$	Ratio $\alpha 1/\alpha 2$	$\alpha 1$	$\alpha 2$	Ratio $\alpha 1/\alpha 2$	$\alpha 1$	$\alpha 2$	Ratio $\alpha 1/\alpha 2$	
Control	62.3	30.9	2.02	53.5	27.3	1.96	73.2	7.9	9.3	
1-Hydroxyethane-1,1-diphosphonate	65.9	28.8	2.26	44.2	22.8	1.94	72.2	7.6	9.5	
Dichloromethanediphosphonate	61.9	32.5	1.90	54.5	22.8	2.39	84.7	6.8	12.5	

from those obtained *in vitro*. Bone cells *in vitro* were shown to be insensitive to 1-hydroxyethane-1,1-diphosphonate; however, when tested *in vivo* this diphosphonate inhibited the incorporation of [³H]-proline into collagen by 48 and 30% for calvaria and tibial bone shafts respectively. No effect on cartilage of the tibial heads by 1-hydroxyethane-1,1-diphosphonate was apparent.

Effect in vivo of diphosphonate on collagen type synthesized by bone and cartilage

Studies on the type of collagen synthesized by

bone and cartilage under the influence of diphosphonates revealed that neither diphosphonate influenced the tissue specific collagen type in any way (Table 8).

Discussion

The results of the present investigation demonstrate that isolated articular chondrocytes cultured in the presence of dichloromethanediphosphonate synthesize elevated amounts of collagen. This conclusion is based on data that indicate that the

incorporation of [^3H]proline into newly synthesized collagen recovered from the cell layer as well as that isolated from the growth medium was increased by a substantial margin (Table 1). With the utilization of chemical analysis to determine the actual amount of collagen synthesized and secreted into growth medium, it became clear that the extent of the stimulation, although still considerable, was somewhat less pronounced, as initially indicated by the production of radioactively labelled collagen (Table 2). The apparent discrepancy in the above-mentioned results may be explained by the fact that dichloromethanediphosphonate-treated articular chondrocytes take up amino acids from the growth medium to a significantly greater extent than do the untreated control cells (Guenther *et al.*, 1979). This increased cellular amino acid uptake undoubtedly will cause the synthesis of a collagen that is marked by a high specific radioactivity ([^3H]proline/molecule of collagen).

The increment in the synthesis of collagen, with the concomitant increase in proteoglycans (Guenther *et al.*, 1979), suggests that total extracellular matrix formation became enhanced by the influence of this particular diphosphonate. Likewise, these findings provide reason to speculate that the action of this compound on articular chondrocytes may not be confined to a single cellular event, but to a variety of different biochemical reactions.

It was noteworthy that 1-hydroxyethane-1,1-diphosphonate failed to affect the synthesis of collagen in chondrocytes. This finding appears to be in accordance with previous results, where it was found that the synthesis of proteoglycans was likewise not affected by 1-hydroxyethane-1,1-diphosphonate (Guenther *et al.*, 1979). The ineffectiveness of this diphosphonate on chondrocyte matrix formation suggests that the intrinsic biological activity of a diphosphonate depends, in all probability, not only on the P-C-P arrangement, common to all geminal diphosphonates, but on the kind of side groups that are chemically attached to the molecule. Any inference as to the biological action of pyrophosphate, marked by P-O-P, on extracellular matrix formation should thus be avoided.

At the present stage of our knowledge it is difficult to know or even to predict by which mechanism dichloromethanediphosphonate acts on chondrocytes to stimulate collagen biosynthesis. However, it should be pointed out that the cells apparently have to be exposed to the diphosphonate for more than 24 h in order to respond. These results are in sharp contrast with those of studies performed by Guenther *et al.* (1979), where it was shown that the synthesis of proteoglycans became stimulated within 24 h after addition of the diphosphonate to the growth medium. Furthermore, the fact that the action of dichloromethanediphosphonate on

the synthesis of collagen persisted only throughout three cell passages suggests that the ability of the cells to respond to the diphosphonate apparently becomes finite after a prolonged treatment (Table 5).

Since most of available data on the biological action of diphosphonates are on studies pertaining to bone, we considered it worthwhile to examine whether bone cells exposed to dichloromethanediphosphonate would likewise alter the synthesis of collagen. The results (Table 6) seem to indicate that such was indeed the case. However, the stimulatory effect was noticed only with collagen accumulated in the growth medium and not with collagen associated with the cell layer.

The studies on the biosynthesis of collagen *in vivo* (Table 7) indicate that the effect is also present when dichloromethanediphosphonate is administered to living animals, thus suggesting that the effect is not a phenomenon produced only *in vitro*. This increase in [^3H]proline incorporation was found to occur even though it was shown previously that dichloromethanediphosphonate *in vivo* was inhibitory to osteoblast cell proliferation (Miller & Jee, 1977). An increase in cellular uptake of [^3H]proline provoked by dichloromethanediphosphonate, as was shown to occur with chondrocytes (Guenther *et al.*, 1979), can be dismissed, since no such effect was obtained with bone cells (R. Felix & H. Fleisch, unpublished work). On the basis of a lack of increased radioactivity with the bone cell layer (Table 6), along with the fact that isolated bone cells constitute a cell population that in its composition is heterogeneous, containing both osteoblast- and osteoclast-like cells (Luben *et al.*, 1976), it appears conceivable that the apparent increase in radiolabelled bone collagen found *in vivo* and *in vitro* is not entirely due to an elevation of collagen production, but to a partial inhibition of collagen breakdown. Since dichloromethanediphosphonate was shown to be toxic towards bone cells (Table 5), it is possible that the osteoclast-like cells found in culture or the osteoclast in living bone may be especially subject to this toxic effect. Studies *in vivo* and *in vitro* have shown that dichloromethanediphosphonate acts as a potent inhibitor on osteoclastic bone resorption (Fleisch *et al.*, 1969; Russell *et al.*, 1970; Reynolds *et al.*, 1972).

Further experiments are needed in order to determine by what mechanism dichloromethanediphosphonate acts on bone cells to increase net collagen production.

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