Effects of 1-Hydroxyethane-1,1-diphosphonate and Dichloromethanediphosphonate on Rabbit Articular Chondrocytes in Culture

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Investigations were carried out to assess the effects of disodium 1-hydroxyethane-1,1-diphosphonate and disodium dichloromethanediphosphonate (compounds containing a P–C–P bond) on isolated rabbit articular chondrocytes in culture. Studies on growth behaviour showed that both diphosphonates displayed inhibitory actions, dichloromethanediphosphonate producing the larger effect. Both compounds inhibited the uptake of 2-deoxy-D-glucose, dichloromethanediphosphonate once more being the more potent of the two. The uptake of α -aminoisobutyrate was considerably increased by chondrocytes treated with dichloromethanediphosphonate, whereas 1-hydroxyethane-1,1-diphosphonate showed no effects. The biosynthesis of sulphated extracellular macromolecules secreted by the cells into the pericellular space as well as into the growth medium was greatly increased by dichloromethanediphosphonate but not by 1-hydroxyethane-1,1-diphosphonate. The stimulatory effect was dose-dependent. Short-term exposure of already confluent cells to dichloromethanediphosphonate as opposed to growing the cells to confluence in the presence of the diphosphonate revealed that the stimulatory effects were already evident after 24 h, indicating that cell division is not necessarily required to produce the observed effects. The increment in proteoglycan synthesis was still evident with cells that were exposed continuously to the diphosphonate in primary as well as secondary culture. Pulse-chase experiments together with studies on the enzyme arylsulphatase revealed that the appearance of increased amounts of proteoglycans was the result of a stimulation in synthesis and not due to an inhibition in turnover.

The diphosphonates are a group of synthetic phosphates characterized by a P–C–P bond and therefore structurally related to the naturally occurring pyrophosphate, which contains a P–O–P bond. For some time now, pyrophosphate has been implicated in the events that lead to the regulation of tissue mineralization. It has been shown that pyrophosphate inhibits both the formation (Fleisch & Bisaz, 1962) and dissolution (Fleisch *et al.*, 1966) of hydroxyapatite crystals *in vitro*. *In vivo* pyrophosphate prevents ectopic calcification (Schibler *et al.*, 1968) and may decrease bone resorption (Orimo *et al.*, 1969; Rasmussen *et al.*, 1970) when administered parenterally. The close structural similarity of the di-

phosphonates to pyrophosphate and their resistance towards enzymic hydrolysis has led to studies of the actions of these compounds on connective and other tissues.

Of the various diphosphonates studied, 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate have been shown to exhibit high biological activity. 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*, 1-hydroxyethane-1,1-diphosphonate being more effective than dichloromethanediphosphonate on the inhibition of hydroxyapatite formation (Jung *et al.*, 1973). *In vivo* high doses of 1-hydroxyethane-1,1-diphosphonate but not of dichloromethanediphosphonate inhibit the accretion of mineral in 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; Russell *et al.*, 1970; Reynolds *et al.*, 1972). On the basis of the work cited above and other experimental research, 1-hydroxyethane-1,1-diphosphonate after extensive clinical trials has become rather useful in the treat-

1-Hydroxyethane-1,1-diphosphonic acid

Dichloromethanediphosphonic acid

ment of various calcium disorders in man, e.g. Paget's disease or myositis ossificans progressiva (Fleisch & Russell, 1977).

Although it appears to be firmly established that the diphosphonates by their physicochemical interaction with Ca^{2+} and hydroxyapatite interfere with the events of tissue mineralization, much has yet to be done to investigate their action(s) on cell metabolism and extracellular organic matrix formation.

Studies *in vivo* (Miller & Jee, 1977; Guenther *et al.*, 1977a) and also investigations performed *in vitro* (Guenther *et al.*, 1977b,c; Howell & Pita, 1977; Fast *et al.*, 1978) have revealed that bone, cartilage and skin cells respond profoundly to a prolonged exposure to diphosphonates.

In a study on experimental arthritis in rats diagnosed as such after the administration of adjuvant into the joints of the animals, 1-hydroxyethane-1,1-diphosphonate was found to prevent periarticular calcification and to inhibit articular changes including cartilage erosion and juxta-articular bone loss (Francis *et al.*, 1972). In view of this report experiments were designed to examine the action of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on isolated articular chondrocytes. The present study acquired further importance by the finding (Guenther *et al.*, 1977a) that dichloromethanediphosphonate supports the biosynthesis of bone and epiphyseal cartilage collagen *in vitro*.

Evidence is presented below which shows (i) that cell growth as well as the uptake of glucose and amino acids by these cells have become altered by the action of either diphosphonate and (ii) that proteoglycan biosynthesis was enhanced by dichloromethanediphosphonate but not by 1-hydroxyethane-1,1-diphosphonate.

Experimental

Materials

Ham's F-12 medium (Ham, 1965), Dulbecco's modified Eagle's medium (Dulbecco & Freeman, 1959), foetal calf serum and penicillin/streptomycin solution were all purchased from Seromed, Fakola A.G., Basel, Switzerland. Trypsin (type III), glutamine and α -aminoisobutyric acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Hyaluronidase was supplied by ICN Pharmaceuticals, Cleveland, OH, U.S.A. Collagenase (type II) was obtained from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Phenylmethanesulphonyl fluoride and ϵ -amino-n-hexanoic acid were obtained from Calbiochem A.G., Lucerne, Switzerland. Dichloromethanediphosphonate and 1-hydroxyethane 1,1-diphosphonate were gifts from Procter and Gamble Co., Cincinnati, OH, U.S.A. Guan-

dinium hydrochloride (ultra pure) was purchased from Schwarz-Mann, Orangeburg, NY, U.S.A. The radioactive compounds D-[6(n)- ^3H]glucosamine hydrochloride (5–15 Ci/mmol), L-[G- ^3H]serine (1–5 Ci/mmol) and α -amino[1- ^{14}C]isobutyric acid (2–10 mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A., and 2-deoxy-D-[1- ^3H]glucose (15–25 Ci/mmol) and $\text{Na}_2^{35}\text{SO}_4$ (carrier-free, 100 mCi/mmol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Triton X-100 and 2-(4-t-butylphenyl)-5-(biphenyl-4-yl)-1-oxa-3,4-diazole (butyl-PBD) used in liquid-scintillation counting were purchased from Christ A.G., Aesch, Switzerland, and CIBA-GEIGY, Basel, Switzerland, respectively. All other chemicals and reagents were of analytical grade and purchased from E. Merck A.G., Darmstadt, West Germany.

Isolation and culture of chondrocytes

Chondrocytes were released by enzymic digestion from slices of articular cartilage taken from the proximal femoral surfaces of 3-month-old KUN FI Hybrid Rex rabbits (supplied by Kunath Futter A.G., Aarau, Switzerland). The digestions were performed as described elsewhere (Benja *et al.*, 1977). Briefly, dissected cartilage slices, after having been washed with Gey's balanced salt solution (Gey & Gey, 1936), were sequentially treated with 10 ml of 0.05% hyaluronidase for 10 min and 4 ml of 0.2% trypsin for 15 min, washed twice with 40 ml of Gey's balanced salt solution and then digested with 4 ml of 0.2% collagenase for 30 min. The enzyme treatments were carried out under CO_2/air (1:19) at 37°C. The cells released from each of these digestions were discarded. The remaining tissue slices were placed in 10 ml of Dulbecco's modified Eagle's medium containing 4500 mg of glucose/l, 10% (v/v) foetal calf serum and 1% penicillin/streptomycin solution, to which was added 2 ml of 0.2% collagenase in Gey's balanced salt solution. The digestion was allowed to proceed for 15 h under CO_2/air (1:19) at 37°C. This procedure releases approx. 90% of the chondrocytes present in the original cartilage slices. Undigested tissue and/or cell aggregates were removed by passing the suspension through a Nitex filter. The cells were harvested by centrifugation and plated at 1.5×10^5 cells per culture dish (60 mm $\times$ 15 mm; Corning Glass Works, Corning, NY, U.S.A.). The chondrocytes were allowed to attach to the culture dish in Ham's F-12 medium (3.5 ml) supplemented with 10% (v/v) foetal calf serum and 1% penicillin/streptomycin solution. After cell attachment, which occurred by 24 h, fresh medium was added which contained either 250 μM -dichloromethanediphosphonate or 250 μM -1-hydroxyethane-1,1-diphosphonate. The cells were grown in the diphosphate-containing medium until confluence,

which generally occurred at the seventh day of culture. Growth medium was changed every second day.

Biosynthesis of proteoglycans

In experiments designed to study the biosynthesis of sulphated proteoglycans in the presence of dichloromethanediphosphonate or 1-hydroxyethane-1,1-diphosphonate, newly confluent chondrocytes were incubated for 24h in Ham's F-12 medium (3.5ml) containing 10% (v/v) foetal calf serum, either 250 μ M-1-hydroxyethane-1,1-diphosphonate or 250 μ M-dichloromethanediphosphonate, 100 μ g of ascorbic acid/ml, 10 μ Ci of L-[G-³H]serine/dish or 10 μ Ci of D-[6(n)-³H]glucosamine/dish, 5 μ Ci of Na₂³⁵SO₄/dish and 1% penicillin/streptomycin solution. The incubation was carried out in CO₂/air (1:19) at 37°C. To determine the distribution of newly synthesized labelled proteoglycans deposited into the cell layer and secreted into the growth medium, the latter was carefully removed. The cell layer was rinsed twice with 2ml of ice-cold 0.9% NaCl and the wash was pooled with the medium. The cell layer was extracted for 24h at 4°C with 4M-guanidinium hydrochloride in 0.05M-sodium acetate buffer, pH 5.8 (3ml/dish), containing the following proteinase inhibitors: 0.01M-Na₂EDTA, 0.5mM-phenylmethanesulphonyl fluoride and 0.5mM- ϵ -amino-n-hexanoic acid. The extract was then centrifuged at 23000g for 10min, and the resulting pellet was discarded. The clarified extracts were then dialysed against 0.5M-guanidinium hydrochloride in 0.05M-sodium acetate buffer, pH 5.8, containing the proteinase inhibitors for 24h at 4°C. To the dialysed samples were added 40 μ g of carrier chondroitin sulphate (Sigma)/ml, 10 μ l of a saturated sodium acetate solution and enough 100% ethanol to obtain a final concentration of 80% (v/v). After 2h at 4°C the precipitate was dissolved in 0.1M-sodium acetate buffer, pH 5.5. Samples were taken for determinations of radioactivity, protein content and the amount of sulphated glycosaminoglycans. The medium, after the addition of 40 μ g of carrier chondroitin sulphate/ml, was extensively dialysed against 0.1M-(NH₄)₂SO₄ and then distilled water.

The proteoglycans released into the growth medium by the chondrocytes were precipitated by adding to the dialysed culture medium cetylpyridinium chloride to a final concentration of 0.1%. The precipitate was dissolved with 2M-NaCl and reprecipitated with 4 vol. of 100% ethanol. The resulting precipitate was redissolved in 0.1M-sodium acetate and used for the determination of radioactivity.

Determination of glycosaminoglycans

The isolation of glycosaminoglycans was carried out by the procedure of Dorfman & Ho (1970).

Proteoglycans isolated from the chondrocyte cell layer or from the growth medium were dissolved in 3ml of 0.1M-sodium acetate buffer, pH 5.5, containing 5mM-cysteine and 5mM-EDTA. After addition of 6mg of papain/ml the mixture was incubated for 36h at 65°C. The incubation was terminated by the addition of trichloroacetic acid to a final concentration of 5% (w/v). The resulting precipitate was removed by centrifugation, and the supernatant was dialysed extensively against distilled water. The glycosaminoglycans were precipitated by adding cetylpyridinium chloride, redissolved in 2M-NaCl, reprecipitated with 80% (v/v) ethanol and redissolved in 0.1M-sodium acetate buffer. Samples were taken for the determination of radioactivity.

Determination of (a) sugar uptake and (b) amino acid uptake by diphosphonate-treated cells

(a) *Sugar uptake.* The uptake of sugar by confluent chondrocytes cultured in the presence or absence of diphosphonates was determined by using 2-deoxy-D-glucose. The cell layer, after removal of the growth medium, was rinsed twice with 3ml of Hanks minimal essential medium (Eagle, 1959) without glucose or diphosphonates. The cells were then cultured for a designated period of time at 37°C in Hanks medium containing 1 μ Ci of 2-deoxy-D-[1-³H]glucose/ml, 1mM non-radioactive 2-deoxy-D-glucose and either 250 μ M-1-hydroxyethane-1,1-diphosphonate or 250 μ M-dichloromethanediphosphonate. The incubation was terminated by aspirating off the medium containing the radiolabelled sugar and rinsing the cell layer three times with 3ml of ice-cold Hanks medium. The cells were digested with 0.3M-KOH for 1h at 37°C. Samples were taken for DNA determination (Cerriotti, 1952) and for liquid-scintillation counting of radioactivity. The amount of intracellular phosphorylated sugar was measured by the BaSO₄ procedure (Kletzien & Perdue, 1973). Briefly, a neutralized sample of the cell digest was diluted to 0.5ml with distilled water, and 0.2ml of 0.3M-Ba(OH)₂ was added followed by 0.2ml of 5% (w/v) Na₂SO₄. The precipitate was removed by centrifugation, and the quantity of free tritiated 2-deoxy-D-glucose in the supernatant was determined. The amount of phosphorylated sugar is given by the difference between the quantity present in the cell extract and the quantity of free 2-deoxy-D-glucose.

(b) *Amino acid uptake.* The procedure used was essentially the same as described under (a), except that the incubation medium contained 3 μ Ci of α -amino[1-¹⁴C]isobutyric acid/ml, 1mM non-radioactive α -aminoisobutyric acid, 1000mg of glucose/l and either 250 μ M-1-hydroxyethane-1,1-diphosphonate or 250 μ M-dichloromethanediphosphonate. Determinations were run in triplicate.

Preparation of chondrocyte subcultures

Chondrocytes in monolayer were brought into suspension by incubating the cultures for 30–40 min at 37°C in CO₂/air (1:19) in a solution containing 0.2% trypsin, 400mg of KCl/l, 6800mg of NaCl/l, 2200mg of NaHCO₃/l, 1000mg of glucose/l and 5mg of Phenol Red/l. The cells were harvested by centrifugation at 400g for 7 min. The resulting cell pellet was resuspended in Dulbecco's modified Eagle's medium, plated at a density of 1.5×10^5 cells/dish (3.5ml) and grown to confluence in the above-mentioned culture medium.

Cell counts

The cells were suspended as described under 'Preparation of chondrocyte subcultures'. Cell counts were done in Dulbecco's modified Eagle's medium with a Coulter counter (model Instrudial D; Coulter Electronics, Dunstable, Beds., U.K.).

Biochemical methods

(a) Arylsulphatase A (EC 3.1.6.1) activity was determined by the method described by Barrett (1972) with 4-nitrocatechol sulphate as substrate. The incubation mixture contained 4mM-4-nitrocatechol sulphate, 0.125M-sodium acetate buffer, pH4.7, and cell extract in a final volume of 0.2ml. The mixture was incubated for 1h at 37°C and its absorbance read at 513 nm.

(b) DNA was prepared (Halliburton & Thomson, 1965) and determined (Cerriotti, 1952) with the use of indole.

(c) Protein was determined by the method of Lowry *et al.* (1951).

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

All results given in this paper are for one representative experiment from a minimum of three experiments.

Results

Effect of diphosphonates on cell growth

Equal numbers of articular cartilage cells were plated and grown in media with or without 250 μ M-1-hydroxyethane-1,1-diphosphonate or 250 μ M-dichloromethanediphosphonate. Cell growth was allowed to proceed until cell confluence was obtained, which occurred on the average after 7 days, as shown in Fig. 1. Daily cell counts, which were carried out from the third to the tenth day, revealed that there was a slight inhibition of growth in the cultures to which diphosphonates had been added. The inhibition was already detectable after the third day and

Fig. 1. Growth curve of rabbit articular chondrocytes in the presence and absence of diphosphonates

The 60000 cells were plated in 6cm-diameter dishes on day 0. Then 250 μ M-1-hydroxyethane-1,1-diphosphonate and 250 μ M-dichloromethanediphosphonate were added on day 1. Cell counts from day 3 to day 10 were done with a Coulter counter. Each experimental point on the graph represents the mean for five separate dishes; S.E.M. values were less than $\pm 4\%$ and therefore are not included in the Figure. ■, Control; ●, +250 μ M-1-hydroxyethane-1,1-diphosphonate; ▲, +250 μ M-dichloromethanediphosphonate.

reached maxima of 9% and 18% for 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate respectively on day 10.

Influence of diphosphonates on 2-deoxy-D-glucose uptake by chondrocytes

The amounts of total uptake of 2-deoxy-D-glucose and formation of its phosphorylated derivative at various time intervals are illustrated in Fig. 2. At a concentration of 1 mM-2-deoxy-D-glucose, control cells as well as those cultured in the presence of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate took up the sugar in a linear fashion for 20min. The phosphorylation of 2-deoxy-D-glucose paralleled the uptake during this time. Both diphosphonates inhibited the cellular uptake of this sugar derivative as compared with the control cells, dichloromethanediphosphonate showing a greater effect than 1-hydroxyethane-1,1-diphosphonate. Decreases of 20% and 10% in 2-deoxy-D-glucose uptake were observed for dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate respectively. The amounts of free intracellular sugar as determined by the difference of total and phosphorylated 2-deoxy-D-glucose appeared to be identical in the control and in the di-

Fig. 2. Time-course study of the uptake of 2-deoxy-D-glucose by chondrocytes treated with diphosphonates

Cells grown to confluence in the presence and absence of dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate were exposed to 1 mM-2-deoxy-D-glucose. Intracellular total 2-deoxy-D-glucose and phosphorylated 2-deoxy-D-glucose were measured with and without the diphosphonates at time intervals of 1, 2, 5, 10 and 20 min. ■, ● and ▲, Total intracellular 2-deoxy-D-glucose; □, ○ and △, phosphorylated 2-deoxy-D-glucose. (a) Control; (b) +250 μ M-1-hydroxyethane-1,1-diphosphonate; (c) +250 μ M-dichloromethanediphosphonate. Experimental points represent the means for three separate dishes. The S.E.M. values are not drawn because they are smaller than the symbols.

phosphonate-treated cells. These data suggest that the action of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on the uptake of 2-deoxy-D-glucose and possibly of glucose and other sugars may be on the enzyme(s) catalysing the phosphorylation reaction(s) and probably not at the plasma-membrane level.

Influence of diphosphonates on amino acid uptake by chondrocytes

The time course of the uptake of α -aminoisobutyrate is shown in Fig. 3. The dichloromethanediphosphonate-treated cells accumulated this synthetic amino acid to a significantly greater extent than did the untreated control cells. The uptake proceeded in a linear fashion over a 20 min time span. Cells that were exposed to 1-hydroxyethane-1,1-diphosphonate did not show an increased α -aminoisobutyrate uptake; on the contrary a small, but not statistically significant, decrease in uptake was observed.

Effects of diphosphonates on the biosynthesis and secretion of 35 S-labelled glycosaminoglycans

The rate of synthesis of 35 S-labelled glycosaminoglycans was followed by the incorporation of $^{35}\text{SO}_4^{2-}$ into macromolecular anionic products secreted and deposited partly into the extracellular matrix surrounding the cell and into the culture medium by

Fig. 3. Time-course study of the uptake of α -aminoisobutyrate by chondrocytes treated with diphosphonates

The time-dependent uptake study was carried out with diphosphonate (250 μ M)-treated cells in culture media containing 1 mM- α -aminoisobutyrate and the appropriate diphosphonate. The intracellular amount of the amino acid analogue measured is expressed in nmol/ μ g of DNA. Experimental points are means $\pm$ S.E.M. for three separate dishes. ■, Control; ●, +250 μ M-1-hydroxyethane-1,1-diphosphonate; ▲, +250 μ M-dichloromethanediphosphonate.

chondrocytes that were or were not treated with dichloromethanediphosphonate. During the first 10 h of labelling, sulphated glycosaminoglycans

associated with the cell layer accumulated almost linearly with time, as shown in Fig. 4(a). During the next 48 h the rate of synthesis increased markedly and then declined, the extent of incorporation of $^{35}\text{SO}_4^{2-}$ reaching a plateau value by 72 h. Throughout the entire time course, dichloromethanediphosphonate-treated cells incorporated $^{35}\text{SO}_4^{2-}$ at a considerably higher rate than did the untreated control cells. The rate of secretion of labelled glycosaminoglycans into the culture, shown in Fig. 4(b), was also similarly increased by dichloromethanediphosphonate. The appearance of the labelled macromolecules in the culture medium roughly paralleled the appearance of those associated with the cell layer.

To substantiate further the effects of the diphosphonates, cells were cultured in different concentrations of dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate. To determine whether glucosamine incorporation followed the same pattern of incorporation as $^{35}\text{SO}_4^{2-}$, cells were labelled simultaneously with $\text{Na}_2^{35}\text{SO}_4$ and $[^3\text{H}]$ -glucosamine. A clear dose-related response of $^{35}\text{SO}_4^{2-}$ incorporation was observed with dichloromethanediphosphonate, as shown in Table 1. The incorporation of tritiated glucosamine into the macromolecules did not quite follow the pattern shown by $^{35}\text{SO}_4^{2-}$; whereas the $^{35}\text{SO}_4^{2-}$ incorporation decreased with decreasing concentrations of dichloromethanediphosphonate, a moderate enhancement in D- $[^3\text{H}]$ glucosamine incorporation was observed. At a concentration of $2.5\mu\text{M}$ no change in incorporation of either radiolabelled precursor was seen. 1-Hydroxyethane-1,1-diphosphonate at a concentration of $250\mu\text{M}$ did not have any statistically significant effect on the incorporation of either radiolabelled precursor.

To ascertain whether dichloromethanediphosphonate truly increased the synthesis or may possibly have displayed inhibitory action on the degradation of the glycosaminoglycans, pulse-chase studies were carried out. Cells were labelled for 24 h with $\text{Na}_2^{35}\text{SO}_4$ and $[^3\text{H}]$ glucosamine followed by 48 h of chase in medium containing non-radioactive Na_2SO_4 and glucosamine. After the chase the cell layer was examined for $^{35}\text{SO}_4^{2-}$ and $[^3\text{H}]$ glucosamine-labelled glycosaminoglycans. The results of this experiment, as depicted in Table 2, show that glycosaminoglycans labelled not only with $^{35}\text{SO}_4^{2-}$ but also with $[^3\text{H}]$ -glucosamine did not lose any significant amount of radioactivity. The amount of radioactivity released into the medium from the diphosphonate-treated and control cells by the chase appeared to be statistically insignificant and thus negligible, indicating that the increase in total glycosaminoglycans observed in the presence of dichloromethanediphosphonate is probably due to increased synthesis and not to an inhibition of degradation. Furthermore, the enzyme

Fig. 4. Incorporation of $^{35}\text{SO}_4^{2-}$ into glycosaminoglycans isolated from the cell layer (a) and medium (b).

Chondrocytes grown to confluence in the presence or absence of $250\mu\text{M}$ -dichloromethanediphosphonate were allowed to incorporate $^{35}\text{SO}_4^{2-}$ for the indicated periods of time. After papain digestion and dialysis ^{35}S -labelled glycosaminoglycans were isolated as described in the Experimental section. The amount of total glycosaminoglycans recovered from the cell layer is shown in (a) and that from medium in (b). Each experimental point represents the mean $\pm$ S.E.M. for five separate dishes. ■, Control; ▲, + $250\mu\text{M}$ -dichloromethanediphosphonate.

activity of arylsulphatase measured in dichloromethanediphosphonate-treated and control cultures showed no detectable differences, supporting the

Table 1. *Biosynthesis of sulphated glycosaminoglycans in the absence and presence of diphosphonates*

Chondrocytes grown to confluence under the influence of 1-hydroxyethane-1,1-diphosphonate and various concentrations of dichloromethanediphosphonate were pulsed with 20 μCi of D-[6(n)- ^3H]glucosamine/ml and 5 μCi of $\text{Na}_2^{35}\text{SO}_4$ /ml for 24 h. Glycosaminoglycans were prepared and isolated as described in the Experimental section. Radioactivity incorporated into newly synthesized glycosaminoglycans is given in c.p.m./ μg of DNA. Results are expressed as means $\pm$ S.E.M., the numbers of dishes being given in parentheses.

Diphosphonate	Concn. (μM)	Radioactivity incorporated (c.p.m./ μg of DNA)		DNA (μg /dish)
		[^3H]Glucosamine	$\text{Na}_2^{35}\text{SO}_4$	
Control	—	2408 $\pm$ 77	3553 $\pm$ 149 (8)	44.03 $\pm$ 0.81 (3)
1-Hydroxyethane-1,1-diphosphonate	250	2520 $\pm$ 57	3881 $\pm$ 101* (10)	39.67 $\pm$ 1.01‡ (3)
Dichloromethanediphosphonate	250	2653 $\pm$ 55‡	4648 $\pm$ 102¶ (8)	38.58 $\pm$ 0.79 (3)
Dichloromethanediphosphonate	25	2768 $\pm$ 36¶	4384 $\pm$ 52¶ (8)	43.07 $\pm$ 0.43 (3)
Dichloromethanediphosphonate	2.5	2533 $\pm$ 118	3702 $\pm$ 153 (7)	43.23 $\pm$ 0.57 (3)

* Not statistically significant.

‡ $P < 0.02$ (control versus diphosphonate).

|| $P < 0.005$ (control versus dichloromethanediphosphonate).

¶ $P < 0.001$ (control versus dichloromethanediphosphonate).

Table 2. *Effect of dichloromethanediphosphonate on the turnover of glycosaminoglycans associated with the cell layer*

Chondrocytes were grown to confluence without or with 250 μM -dichloromethanediphosphonate. After a labelling period of 24 h with [^3H]glucosamine and $^{35}\text{SO}_4^{2-}$ fresh medium without the radioactive precursors but with diphosphonates was added to half of the culture dishes. The determination of radioactivity chased from the cell layer into the medium was carried out as described in the Experimental section. Results are given in c.p.m./ μg of DNA before (a) and after the chase (b), and are expressed as means $\pm$ S.E.M., the numbers of dishes being given in parentheses.

	Radioactivity incorporated (c.p.m./ μg of DNA)	
	[^3H]Glucosamine	$\text{Na}_2^{35}\text{SO}_4$
Control		
(a) Before	863 $\pm$ 99 (5)	1078 $\pm$ 206 (5)
(b) After	832 $\pm$ 108	891 $\pm$ 216
Dichloromethane- diphosphonate		
(a) Before	1415 $\pm$ 108 (5)	2500 $\pm$ 123 (5)
(b) After	1187 $\pm$ 142	2220 $\pm$ 198

concept of stimulation of glycosaminoglycan synthesis (H. L. Guenther, H. E. Guenther & H. Fleisch, unpublished work).

Short-term effect of dichloromethanediphosphonate on the biosynthesis of glycosaminoglycans by chondrocytes

In the experiments described above chondrocytes were cultured in the presence of the diphosphonates for 8–10 days before glycosaminoglycan synthesis was determined. To determine the short-term effect

of dichloromethanediphosphonate, [^3H]glucosamine, $\text{Na}_2^{35}\text{SO}_4$ and the diphosphonate were all added at the same time to confluent cultures, and after 24 h the synthesis of glycosaminoglycans was measured. The data in Table 3 show that the $^{35}\text{SO}_4^{2-}$ incorporation into the glycosaminoglycans was considerably higher than in control experiments even when the cells were exposed to dichloromethanediphosphonate for only 24 h. However, the increase was lower than when the cells had been exposed to this drug for 10 days. This effect was observed for glycosaminoglycans isolated from the cell layer as well as from the culture medium. The incorporation of D-[^3H]glucosamine by the cells exposed to dichloromethanediphosphonate for 24 h was also considerably greater than that by non-treated cells. Furthermore, as shown in the last column of Table 3, the DNA values were very similar to those obtained for the controls, indicating that dichloromethanediphosphonate at a concentration of 250 μM caused no cell death during the 24 h incubation time.

Effects of diphosphonates on the ability of the cells to incorporate [^3H]serine into the protein core of proteoglycan aggregates

Muir (1958) had already suggested that serine residues were involved in the linkage of glycosaminoglycans to protein, and subsequently the work of Rodén & Smith (1966), Gregory *et al.* (1964) and Anderson *et al.* (1965) elucidated the detailed structure of chondroitin sulphate linkage region, showing that these sulphated sugar molecules are covalently linked to protein via a serine residue. After having reached confluence, diphosphonate-treated and control cells were labelled for 24 h with [^3H]serine and $\text{Na}_2^{35}\text{SO}_4$ to assess the influence of

1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on the biosynthesis of the core protein and to establish whether a correlation exists between the increased $^{35}\text{SO}_4^{2-}$ incorporation and the biosynthesis of the core protein. The results in Table 4 show that dichloromethanediphosphonate increased the incorporation of ^3H serine into the proteoglycans extracted from the cell layers by guanidinium hydrochloride by 30%. The incorporation of $^{35}\text{SO}_4^{2-}$ in this experiment was 42% above control values. 1-Hydroxyethane-1,1-diphosphonate, however, had no statistically significant effect on the incorporation of either radioactive precursor. Protein determinations performed on the guanidinium hydrochloride extracts of the cell layer revealed that cells exposed to dichloromethane-

diphosphonate contained about 30% more proteins (Table 5). Thus the calculated specific radioactivity (radioactivity incorporated divided by protein concentration) of the glycosaminoglycans produced equal values for control and diphosphonate-treated cells.

Effect of dichloromethanediphosphonate on the incorporation of $^{35}\text{SO}_4^{2-}$ into sulphated glycosaminoglycans by chondrocytes grown in secondary culture in the presence and absence of dichloromethanediphosphonate

To determine (a) whether secondary cultures of chondrocytes respond to dichloromethanediphosphonate, (b) whether secondary cultures obtained from

Table 3. *Biosynthesis of sulphated glycosaminoglycans by chondrocytes to which dichloromethanediphosphonate was introduced (a) after cell attachment and (b) after cells had reached confluence*

Chondrocytes were in one instance (a) exposed to 250 μM -dichloromethanediphosphonate immediately after plated cells had attached to culture dishes, and in the other case (b) medium containing 250 μM -dichloromethanediphosphonate, ^3H glucosamine and $\text{Na}_2^{35}\text{SO}_4$ was added to confluent cells for the labelling period of 24 h. Results are given in c.p.m./ μg of DNA and expressed as means $\pm$ S.E.M., the numbers of dishes being given in parentheses.

	Radioactivity incorporated (c.p.m./ μg of DNA)				
	Cell layer		Medium		DNA ($\mu\text{g}/\text{dish}$)
	^3H Glucosamine	$\text{Na}_2^{35}\text{SO}_4$	^3H Glucosamine	$\text{Na}_2^{35}\text{SO}_4$	
Control	1855 $\pm$ 51	2770 $\pm$ 165 (5)	1658 $\pm$ 62	2008 $\pm$ 78 (5)	57.86 $\pm$ 4.28 (3)
Dichloromethanediphosphonate (a)	2013 $\pm$ 164*	4459 $\pm$ 395 (5)	1925 $\pm$ 96†	4200 $\pm$ 138¶ (5)	42.48 $\pm$ 1.84‡ (3)
Dichloromethanediphosphonate (b)	2308 $\pm$ 128§	3600 $\pm$ 142 (5)	2050 $\pm$ 115‡	3050 $\pm$ 182¶ (5)	53.80 $\pm$ 0.54* (3)

* Not statistically significant.

† $P < 0.05$.

‡ $P < 0.02$.

§ $P < 0.01$.

|| $P < 0.005$.

¶ $P < 0.001$.

Table 4. *Effects of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on the incorporation of ^3H serine and $\text{Na}_2^{35}\text{SO}_4$ into proteoglycans synthesized by chondrocytes in culture*

Chondrocytes that had reached confluence were cultured for 24 h in medium containing either diphosphonate, 20 μCi of L-[^3H]serine/ml and 5 μCi of $\text{Na}_2^{35}\text{SO}_4$ /ml. The incorporation of the radioactive precursors into newly synthesized proteoglycans was determined in cell layers and growth media as described in the Experimental section. Results are given in c.p.m./ μg of DNA and are expressed as means $\pm$ S.E.M., the numbers of dishes being given in parentheses.

	Radioactivity incorporated (c.p.m./ μg of DNA)			
	Cell layer		Medium	
	^3H Serine	$\text{Na}_2^{35}\text{SO}_4$	^3H Serine	$\text{Na}_2^{35}\text{SO}_4$
Control	2224 $\pm$ 49	2239 $\pm$ 71 (5)	974 $\pm$ 15	1239 $\pm$ 16 (5)
1-Hydroxyethane-1,1-diphosphonate	2383 $\pm$ 73*	2469 $\pm$ 76* (5)	1006 $\pm$ 55*	922 $\pm$ 43 (5)
Dichloromethanediphosphonate	2870 $\pm$ 35¶	3181 $\pm$ 11¶ (5)	946 $\pm$ 60*	1735 $\pm$ 63¶ (5)

* Not statistically significant.

¶ $P < 0.001$ (control versus dichloromethanediphosphonate).

Table 5. *Protein content of the guanidinium hydrochloride extract of the chondrocyte cell layer*

Cell layers were extracted with 4M-guanidinium hydrochloride for 24h. Protein concentrations in guanidinium hydrochloride extracts were determined by the method of Lowry *et al.* (1951). The calculated specific radioactivity represents the radioactivity (c.p.m.) of [^3H]serine incorporated into cell-layer proteoglycans divided by the amount of protein determined. Results are expressed as means $\pm$ S.E.M., the number of dishes being given in parentheses.

	Protein ($\mu\text{g}/\mu\text{g}$ of DNA)	$10^{-3} \times$ Specific radioactivity ([^3H]serine c.p.m./ μg of protein)
Control	1.059 ± 0.024 (5)	2.100 ± 0.046
1-Hydroxyethane-1,1-diphosphonate	$1.206 \pm 0.059^*$ (5)	$1.976 \pm 0.059^*$
Dichloromethanediphosphonate	$1.372 \pm 0.033^{\dagger\dagger}$ (5)	$2.091 \pm 0.026^*$

* Not statistically significant.

$\dagger\dagger P < 0.001$ (control versus dichloromethanediphosphonate).

primary cultures grown in the presence of dichloromethanediphosphonate still synthesize increased amounts of glycosaminoglycans and (c) whether cells grown during primary and secondary culture in the presence of dichloromethanediphosphonate keep synthesizing increased amounts of glycosaminoglycans, primary cultures with and without dichloromethanediphosphonate were taken and grown in secondary cultures with and without the diphosphonate. The results of these experiments are illustrated in Fig. 5. It is evident that cells that had been treated with dichloromethanediphosphonate in primary cultures still synthesized increased amounts of glycosaminoglycans when they were grown in secondary cultures (Fig. 5b) as compared with secondary cultures of cells that had never been exposed to dichloromethanediphosphonate (Fig. 5a). It is also apparent that secondary cultures of chondrocytes respond to dichloromethanediphosphonate in the same manner as primary cultures (Fig. 5c).

Discussion

The results of the present investigation demonstrate that isolated articular chondrocytes cultured in the presence of dichloromethanediphosphonate or 1-hydroxyethane-1,1-diphosphonate undergo marked biochemical changes. These diphosphonate-induced alterations were shown to be associated with a partial inhibition of cell growth, a diminished cellular glucose uptake and a dichloromethanediphosphonate-mediated enhancement of amino acid uptake. Most striking, however, was the finding that the biosynthesis and subsequent secretion of sulphated macromolecules into the extracellular space appeared to be greatly increased by dichloromethanediphosphonate but not by 1-hydroxyethane-1,1-diphosphonate.

A diphosphonate-mediated inhibition of cell growth, which in the present study reached values of 18% and 9% for dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate respectively, had already been observed in a previous study

Fig. 5. *Effect of dichloromethanediphosphonate on chondrocyte monolayer in secondary culture*

Chondrocytes were grown in primary culture to confluence in the presence and absence of $250 \mu\text{M}$ -dichloromethanediphosphonate. The monolayers were treated with trypsin and replated at 150000 cells/dish in four groups. Group (a) cells continued to grow without diphosphonate, group (b) cells received dichloromethanediphosphonate in primary culture but were grown to monolayer in secondary culture without the diphosphonate, group (c) cells had not been exposed to dichloromethanediphosphonate in primary culture but were allowed to grow to confluence in the presence of $250 \mu\text{M}$ -dichloromethanediphosphonate in secondary culture, and group (d) cells were allowed to grow with $250 \mu\text{M}$ -dichloromethanediphosphonate in primary culture as well as in secondary culture. $\square$, Radioactivity recovered from the cell layer; $\blacksquare$, radioactivity present in the medium. The results are given as means $\pm$ S.E.M. for seven separate dishes.

(Fast *et al.*, 1978), where values of over 50% for dichloromethanediphosphonate and over 30% for 1-hydroxyethane-1,1-diphosphonate were recorded in experiments with rat calvaria cells. In the same report data were presented showing that rabbit ear cartilage cells followed a normal growth pattern in the presence of either diphosphonate. Thus it is apparent that the magnitude of the inhibition of cell

growth depends not only on the cell type but also on the molecular structure of the individual diphosphonate. At the present we can only speculate about the level at which inhibition of growth occurs. The observed partial inhibition of the uptake by chondrocytes of 2-deoxy-D-glucose, and presumably glucose and other sugars as well, may be directly linked to the partial inhibition of growth. One of the features that distinguish connective-tissue cells such as bone and cartilage cells from most other mammalian cells is the way in which they generate their energy. The large production of lactate even in the presence of O_2 (Borle *et al.*, 1960) indicates that the glycolytic pathway supplies most of the energy these cells require. It seems reasonable to assume that a diminished glucose uptake leading ultimately to cell starvation may have caused a slow-down in cell growth. The mechanism by which the uptake of 2-deoxy-D-glucose is partially inhibited by the diphosphonates is unknown. It is tempting to speculate that the inhibition may occur, not at the cell plasma-membrane level, but on one of the enzymes catalysing the degradation of glucose to lactate. This is compatible with data (Fig. 2) showing that the amount of non-phosphorylated 2-deoxy-D-glucose, which is determined by the difference between total intracellular 2-deoxy-D-glucose and the phosphorylated fraction thereof, appeared to be equal in diphosphonate-treated and control cells.

Concomitantly with the decrease in glucose uptake, an increase in amino acid uptake (α -aminoisobutyrate) was observed with chondrocytes exposed to dichloromethanediphosphonate (Fig. 3). This phenomenon, which was not observed with 1-hydroxyethane-1,1-diphosphonate, suggests that these cells are able to adapt themselves to the situation and switch to an alternative energy source such as amino acids to offset the loss in glucose availability.

Whether the elevated amino acid uptake occurred solely as a consequence of a diminished glucose uptake, therefore arguing against inhibition of cell growth being the result of cell starvation, or whether increased amounts were only taken up to support increased extracellular protein synthesis, cannot be resolved at the present time. It can be said, however, that the proteoglycan biosynthesis was greatly increased by dichloromethanediphosphonate in a dose-dependent fashion (Table 1). With $Na_2^{35}SO_4$ as precursor, the incorporation of ^{35}S into sulphated glycosaminoglycans was enhanced by up to 42% above control values.

In a time-course study where control cells incorporated the $^{35}SO_4^{2-}$ in a similar time-dependent fashion to that reported in the literature for fibroblasts (Sjöberg & Fransson, 1977), the increment in synthesis of glycosaminoglycans, which was already evident after 5h, paralleled the control time curve up to 72h. This was observed with ^{35}S -labelled

glycosaminoglycans recovered from the cell layer (Fig. 4a) as well as those isolated from the growth medium (Fig. 4b). Glycosaminoglycans synthesized by chondrocytes are known to be covalently bound via a serine residue to an extended protein backbone, also called core protein, to form a rod-like macromolecule, the proteoglycan subunit (Hascall & Heinegård, 1975; Rosenberg, 1975). Only small amounts of free unattached polysaccharides exist in mammalian cartilage. Thus an increase in serine incorporation, signifying an augmentation in biosynthesis of the core protein, should parallel the enhancement of the glycosaminoglycan biosynthesis. A 30% increase in $[^3H]$ serine incorporation into isolated proteoglycans with a concomitant increase in chemically measured protein unequivocally shows that an increase in proteoglycan biosynthesis had occurred under the influence of dichloromethanediphosphonate. No change was detectable when 1-hydroxyethane-1,1-diphosphonate was employed; even at concentrations above and below $250\mu M$ this diphosphonate failed to affect the synthesis of proteoglycans to any significant extent. The possibility that dichloromethanediphosphonate is an inhibitor of the turnover of these macromolecules, making an accumulation of radioactive label and protein appear to be a stimulation, can be dismissed, on the basis of results obtained from a pulse-chase experiment (Table 2). No statistical differences between control and treated cells were found in an experiment in which radioactivity associated with proteoglycans was chased from the cell layer into the growth medium. The inability of 1-hydroxyethane-1,1-diphosphonate to stimulate articular chondrocytes to synthesize increased amounts of sulphated proteoglycans provides evidence that the inhibition of cartilage erosion observed in experimental arthritis was probably not due to a diphosphonate-mediated increase in cartilage matrix formation (matrix repair) but instead to a prevention of cartilage tissue destruction caused by an as yet unknown mechanism. The fact that 1-hydroxyethane-1,1-diphosphonate was found to cause inhibition of the biosynthesis of glycosaminoglycans in the rat epiphyseal growth plate (Howell & Pita, 1977; Larsson & Larsson, 1976) demonstrates that cells of different specific tissue origins may respond differently to one and the same diphosphonate.

All results discussed so far were obtained from experiments where the diphosphonates had been present throughout the entire growth period. The addition of dichloromethanediphosphonate to already confluent cells for only 24h instead of 10 days revealed that the stimulatory effect on proteoglycan biosynthesis was already apparent, indicating that the chondrocytes need not to go through cell divisions to produce the described effects (Table 3). The amount of DNA per culture dish, reflecting the

cell population, gave some evidence that a short-term treatment with dichloromethanediphosphonate did not produce cell toxicity.

Finally, chondrocytes grown in primary cultures with dichloromethanediphosphonate continued to produce increased amounts of proteoglycan even after the cells were taken and grown in secondary cultures (Fig. 5), suggesting the persistence of the effects caused by the diphosphonate for a long period of time. Moreover, a prolonged exposure of the cells to the drug in primary culture as well as secondary culture indicated that the cells could tolerate a prolonged treatment with the diphosphonate (Fig. 5). The question whether both these diphosphonates may have changed the chondrocytes to alter their phenotypic expression was investigated in a study aimed to examine the biosynthesis of collagen in the presence and absence of these two compounds. The experiments clearly showed that articular chondrocytes grown in the presence of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate continued to synthesize type 2 collagen in primary as well as secondary culture (Guenther *et al.*, 1977b), thus illustrating that these cells remain true in type.

Whether the increment in the biosynthesis of proteoglycan caused by dichloromethanediphosphonate on articular chondrocytes may find any applications in the treatment of human joint diseases where cartilage destruction prevails remains uncertain. Further studies in laboratories as well as clinics will be needed to provide an answer to this question.

Moreover, the close structural resemblance of the diphosphonates to the natural occurring pyrophosphate and the remarkable similarity in their modes of action on some of the biophysical and/or biochemical events implicated in the events of skeletal mineralization and resorption leads one to speculate that pyrophosphate might be involved in the regulation of the synthesis of connective-tissue extracellular matrix. Whether or not such a link can be found between the action of dichloromethanediphosphonate on articular cartilage cells and pyrophosphate awaits further investigations.

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