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The influence of 1-hydroxyethane-1,1-diphosphonate and dichloromethanediphosphonate on lysine hydroxylation and cross-link formation in rat bone, cartilage and skin collagen

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The effects *in vivo* of dichloromethanediphosphonate and 1-hydroxyethane-1,1-diphosphonate on collagen solubility, hydroxylation of lysine and proline and on the formation of collagen intermolecular cross-links were studied by using rat bone, cartilage and skin tissues. Dichloromethanediphosphonate decreased bone collagen solubility both in acetic acid and after pepsin treatment. Although none of the diphosphonates had any effect on the hydroxylation of proline, dichloromethanediphosphonate, but not 1-hydroxyethane-1,1-diphosphonate, increased the number of hydroxylysine residues in the α-chains of bone, skin and cartilage collagen. The stimulatory effect was dose-dependent. The dichloromethanediphosphonate-mediated increase in hydroxylysine residues in bone and cartilage was manifested in an increase of dihydroxylysinonorleucine, the cross-link that is formed by the condensation of two hydroxylysine residues. The cross-link hydroxylysinonorleucine, a condensation product of hydroxylysine and lysine, on the other hand, was decreased. The total number of intermolecular cross-links was not changed by the diphosphonate.

In the preceding paper (Guenther *et al.*, 1981) it was found that dichloromethanediphosphonate, but not 1-hydroxyethane-1,1-diphosphonate, stimulates cartilage and bone net collagen synthesis both *in vitro* when added to cell cultures and *in vivo* after administration to young growing rats. Experiments designed to examine the collagen synthesized in the presence of dichloromethanediphosphonate showed that no apparent changes were found to have occurred in the tissues' specific type of collagen α-chain composition (Guenther *et al.*, 1981). Other qualitative effects were, however, not investigated.

The purpose of the present paper was to investigate whether these diphosphonates alter in some way the quality of collagen formed. Two of the more prominent chemical modifications that occur on the nascent α-chains are the enzyme-catalysed hydroxylation reactions converting proline and lysine into their respective hydroxylated forms. Both hydroxylated amino acids have been implicated as important factors in the stabilization of the collagen molecule and collagen fibre formation. Although it appears well-established that the major function of hydroxyproline is to provide stability to the triple-helical conformation of collagen (Jimenez *et al.*, 1973; Berg & Prockop, 1973), less is known on the exact function(s) of hydroxylysine residues in the α-chains. It seems, however, that much of the stability of bone and cartilage collagen stems from intermolecular cross-links that are formed by the condensation of two hydroxylysine residues (Miller & Robertson, 1973; Robins *et al.*, 1973). The fact that the *N*-terminal non-helical cross-link regions of the collagen α-chains contain lysine residues that can easily become hydroxylated (Miller *et al.*, 1969; Lane & Miller, 1969; Barnes *et al.*, 1971; Miller, 1973) suggests that a selective hydroxylation of lysine residues could have a profound impact on the chemical configuration of cross-links found at these sites.

We have therefore assessed the effect of two diphosphonates on the solubility of collagen, on the hydroxylation of proline and lysine and on the cross-link formation. The present data indicate that dichloromethanediphosphonate, but not 1-hydroxyethane-1,1-diphosphonate, decreases collagen solubility and enhances the number of existing hydroxylysine residues in bone, skin and cartilage collagen. This increase is accompanied by a qualitative change in reducible cross-links. No effect was found on the hydroxylation of proline with either diphosphonate.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Materials

The materials and chemicals used were obtained as follows. L-[2,3-^{3}H]Proline (20–40Ci/mmol) and NaB^3H_4 (>100μCi/mmol) were from New England Nuclear Corp., Dreieichenhain, Germany, and CNBr was from Aldrich-Europe, E. Lotti S.A., Carouge, Switzerland. Reagents for the polyacrylamide-gel electrophoresis were purchased from Bio-Rad Laboratories, I.G., Zurich, Switzerland. The ion-exchange resin Dowex 50W (X4; 100–200 mesh) (Dow Chemical Co.) was obtained from Serva, Heidelberg, Germany. 1-Hydroxyethane-1,1-dephosphonate and dichloromethanediphosphonate were gifts from Procter and Gamble Co., Cincinnati, OH, U.S.A. Triton X-100 and butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] used in liquid-scintillation counting were supplied by Christ A.G., Aesch, Switzerland, and CIBA–Geigy A.G., Basel, Switzerland, respectively. *o*-Phthalaldehyde, Brij-35 and all other chemicals used in the present study were purchased from E. Merck A.G., Darmstadt, Germany. The determinations of collagen intermolecular cross-links and hydroxylysine were performed on a Labotron Pro-1 amino acid analyser (Kontron Analytic A. G., Zurich, Switzerland) with an Aminco fluorocolorimeter (Aminco Instrument Co., Div. Trovenol, Silver Spring, MD, U.S.A., and a calculating integrator (C-R1A Chromatopack; Shimadzu, Kyoto, Japan).

Methods

Animals. Newborn Wistar rats bred in our Institute were subcutaneously injected for 10 consecutive days with either dichloromethanediphosphonate, 1-hydroxyethane-1,1-diphosphonate or 0.9% (w/v) NaCl. The diphosphonates were administered at a dose of 5, 10 and 20mg of P·(kg of body weight)$^{-1}$ at concentrations of 23.44mg dichloromethanediphosphonate/ml and 20.16mg 1-hydroxyethane-1,1-diphosphonate/ml in physiological saline, pH7.4. Three litters for each respective diphosphonate concentration and one litter to be used as control were required for each experiment. Care was taken so that each litter consisted of an equal number (10) of animals.

Tissue preparation. The preparation of tissues and collagen from the various tissues to be analysed was described in the preceding paper (Guenther *et al.*, 1981). Briefly, calvaria, tibia and skin were cleaned from fascia, adherent cells and cell debris. Bone marrow trapped in the tibial shafts was removed with the aid of a syringe after the tibial heads had been separated from the shafts. The tibial heads, which proved to consist of cartilaginous tissue only, were processed as separate tissues. Calvaria and tibial shafts after having been pulverized with a bone mill using liquid N_2 were demineralized with 0.5M-EDTA, pH7.9 at 4°C. Resulting demineralized bone powder was then washed with deionized water, freeze-dried, hydrolysed and used for determination of hydroxylysine, hydroxyproline and intermolecular cross-links. The cartilaginous tissues from the tibial heads were initially extracted with 4M-guanidinium chloride in 0.05M-sodium acetate buffer, pH5.8, for 48h to remove most of proteoglycans before being subjected to acid hydrolysis. Skin tissue was defatted with acetone before it was submitted to a pepsin/acetic acid extraction (1mg of pepsin in 1ml of 0.5M acetic acid) for 72h at 4°C. Collagen solubilized by pepsin and precipitated by dialysis against deionized water containing 0.01M-disodium phosphate was harvested by centrifugation and subsequently hydrolysed with 6M-HCl.

Solubility determination of collagen synthesized in vivo in rats treated with dichloromethanediphosphonate. Treatment of rats and preparation of explants was performed as described above. Calvaria and tibial shaft after having been pulverized and demineralized were washed with deionized water and subsequently freeze-dried. To determine the initial collagen content of the dried demineralized tissues, portions were acid-hydrolysed and hydroxyproline determination was subsequently performed on the tissue hydrolysates. The main portion of the tissue powder was subjected to acid extraction for 72h with 0.5M-acetic acid. Extracts and residues were separated by centrifugation at 10000*g* for 20min. The acetic acid extract was then acid-hydrolysed for hydroxyproline determination. The acetic acid-insoluble residues were re-extracted with pepsin/acetic acid (1mg of pepsin/ml of 0.5M-acetic acid). The extraction was performed for 24h at 4°C with gentle shaking. Resulting extract was clarified by centrifugation at 22000*g* for 45min and subsequently acid-hydrolysed to determine the amount of collagen solubilized by pepsin.

Determination of hydroxylysine. Collagen isolated from skin, calvaria, tibial shafts and epiphysial cartilage taken from the tibial heads was hydrolysed under N_2 with 6M-HCl in sealed tubes at 108°C for 18h. The quantitative determination of hydroxylysine and lysine was performed on an amino acid analyser equipped with a column (40cm × 5mm) packed with a high-efficiency cation-exchange resin (Durram DC-G6; Durram Chemical Corp., Palo Alto, CA, U.S.A.). A fluorometric detection method, with *o*-phthalaldehyde, was used to quantify both amino acids (Roth, 1976).

Determination of proline and hydroxyproline. The quantitative determination of hydroxyproline to assess the effects of the diphosphonates on the hydroxylation of proline was performed with acid-hydrolysed collagen isolated from tissues that had been excised from the animals and subsequently

cultured in minimum essential medium for 24h in the presence of [^{3}H]proline [see the preceding paper (Guenther *et al.*, 1981)]. The separation of [^{3}H]-proline from [^{3}H]hydroxyproline was achieved on a column (30cm × 0.5cm) packed with Dowex WX-2 cation-exchange resin, which was equilibrated and eluted with 0.2M-sodium citrate buffer, pH2.6.

Digestion of collagen with CNBr. Hydrolysis of purified collagen into fragments with CNBr was performed as described by Scott & Veis (1976).

SDS/polyacrylamide-gel electrophoresis. SDS/polyacrylamide-gel electrophoresis, which resolved the various collagen CNBr digests into the respective 'CNBr peptides', was performed as described by Guenther *et al.* (1977), with the exception that 15 rather than 7.5% (w/w) acrylamide gels were used. The CNBr peptides were stained with Coomassie Blue and scanned at 550nm with a Gilford spectrophotometer equipped with a gel transporter that was synchronized with a recorder.

Determination of intermolecular cross-links. (*a*) Reduction with NaB^3H_4. Pulverized and subsequently demineralized tissues from calvaria, tibia and epiphysial cartilage were suspended in 0.2M-sodium phosphate buffer, pH7.5, in amounts of 10mg of tissue/ml of buffer. After the addition of NaB^3H_4 in a ratio of 200μCi/mg of tissue and enough non-radioactive $NaBH_4$ to make the buffer 1mM, the reduction was allowed to proceed at room temperature for 1h. The reaction was stopped by acidification to pH4.0–4.5 with acetic acid. The tissues were then thoroughly dialysed against deionized water, freeze-dried and subsequently hydrolysed.

(*b*) Determination of reduced cross-links. Equal portions of the various collagen hydrolysates, on the basis of hydroxyproline content determined by the method of Stegemann & Stalder (1967), were applied on to the amino acid analyser. The individual NaB^3H_4-reduced and thus acid-stable cross-links were eluted with a stepped sodium citrate buffer system. The first and second buffers, pH3.07 and 4.25 respectively, were made up with 19.11g of trisodium citrate dihydrate, 1g of phenol and 4ml of a 30% (w/v) Brij-35 solution per litre. The third buffer was comprised of 14.7g of trisodium citrate dihydrate, 37.4g of NaCl, 1g of phenol and 4ml of Brij-35 solution and was adjusted to pH5.2. Fractions were collected at 30s intervals with the aid of a fraction collector. A split-stream device was not required, since it was determined that neither the fluorescent-labelled amino acids nor the reagent gave rise to quench interferences when the same was counted for radioactivity with a liquid-scintillation counter.

Results

Effects in vivo of dichloromethanediphosphonate on the solubility of collagen

The question of whether bone collagen synthesized *in vivo* under the influence of dichloromethanediphosphonate may have altered its solubility characteristics towards mild organic acids or time-limited pepsin extraction is answered in Table 1. The Table shows that bone collagen from diphosphonate-treated rats displayed considerably more resistance to solubilization by 0.5M-acetic acid or pepsin than did bone collagen from control rats. For calvaria, approximately half the amount of collagen was extracted as compared with controls where acetic acid was used. The solubilization of collagen by pepsin was roughly 20% below that of the controls. Similar results were also obtained with collagen from the tibial shafts (Table 1).

Effect of diphosphonates on the hydroxylation of lysine

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Table 1. *Solubility of bone collagen in acetic acid and pepsin solutions of rats treated with dichloromethanediphosphonate*
After rats were treated with dichloromethanediphosphonate, calvaria and tibial shafts were excised, cleaned, demineralized and consequently extracted with 0.5M-acetic acid and pepsin/acetic acid solution (1mg of pepsin/ml of 0.5M-acetic acid). The results below represent those from two independent experiments (roman numerals) with tissue pools of eight to ten animals per experiment.

	Extraction of collagen (% of total)			
	Acetic acid		Pepsin	
Expt. ...	I	II	I	II
Calvaria				
Control	1.88	2.10	16.51	17.12
Dichloromethanediphosphonate	0.99	1.07	12.93	13.17
Tibia				
Control	0.68	0.78	3.45	3.57
Dichloromethanediphosphonate	0.54	0.50	2.87	2.68

Table 2. *Effect in vivo of diphosphonates on the hydroxylation of lysine*
Preparation of tissues and hydroxylysine determination was performed as described in the Experimental section. Results are expressed as hydroxylysine residues/1000 amino acid residues of collagen. Roman numerals denote separate experiments, whereby the data of each experiment were obtained from a pool of eight to ten rats.

		Hydrolysine (residues/1000 residues)			
	Expt.	Calvaria	Tibial heads	Tibial shafts	Skin
Control	I	7.6	15.8	13.1	4.9
	II	8.3	15.9	13.2	—
	III	7.9	16.0	—	—
Dichloromethanediphosphonate-treated					
10mg of $P \cdot kg^{-1}$	I	9.8	19.2	15.3	9.5
	II	10.3	17.9	14.4	9.0
	III	9.6	—	—	—
20mg of $P \cdot kg^{-1}$	I	14.6	20.0	15.6	10.7
1-Hydroxyethane-1,1-diphosphonate-treated					
10mg of $P \cdot kg^{-1}$	I	8.4	—	—	—
20mg of $P \cdot kg^{-1}$	I	7.1	—	—	—

diphosphonates on the hydroxylation of lysine is shown in Table 2. Dichloromethanediphosphonate induced an increase in the number of hydroxylysine residues in collagen from all tissues examined. A doubling of the administered dichloromethanediphosphonate dose from 10 to 20mg of $P \cdot$(kg of body wt.)$^{-1}$ resulted in a still further increase in the number of hydroxylysine residues. No effect was seen with a dose of 1mg of $P \cdot$(kg of body wt.)$^{-1}$ (results not shown). Interestingly enough, no detectable difference in the hydroxylysine content was found in the calvaria between control and 1-hydroxyethane-1,1-diphosphonate-treated rats.

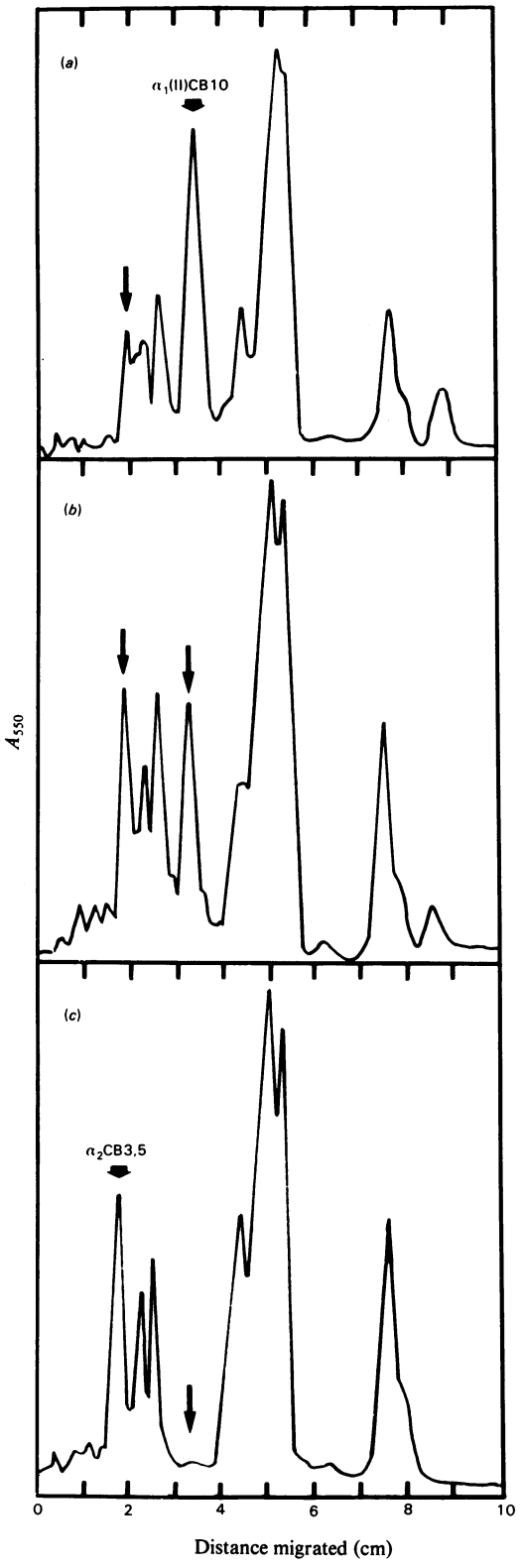
It could be argued that the increase in the hydroxylation of lysine was due to the type of collagen synthesized. Especially, the presence of cartilage type-II collagen, which is known to contain considerably more hydroxylated lysine residues than type-I collagen (Nimni, 1974), may have produced the observed effect. To examine whether such was the case, tibial shafts, tibial heads and calvaria were subjected to CNBr digestion. The tibial heads, which consist mainly of cartilaginous tissue, were analysed in order to have a CNBr peptide pattern specific for type-II collagen. The shafts were digested to determine the amount of cartilage that may have been left after removal of the tibial heads. Fig. 1 depicts the scans of CNBr peptides resolved on SDS/polyacrylamide-gel electrophoresis of collagen derived from these three tissues, all taken from animals that were treated with dichloromethanediphosphonate. The peptide α2CB3,5, characteristic for type-I collagen (bone), appears clearly separated from the α1(II)CB10 peptide, characteristic for type-II collagen. Each peptide gave a stained band with a unique migration distance. Since the calvaria (Fig. 1*c*) did not contain any α1(II)CB10 peptide, it can safely be concluded that this tissue was not contaminated with cartilage type-II collagen, thus providing evidence that dichloromethanediphosphonate has caused the increase of hydroxylysine residues present in the α-chains of bone type-I collagen. Furthermore, as shown in Fig. 1(*b*), collagen analysed from the tibial shafts was obviously mixed with type-II collagen, presumably from the metaphysial cartilage, since both CNBr fragment α1(II)CB10 and fragment α2CB3,5 appear on the gel pattern. The same is true for the shafts. This explains why the control values for hydroxylysine from collagen (Table 1) of the tibial heads were somewhat low (18 residues/1000 amino acids instead of 24, as reported in the literature) and why the values were relatively high for the shafts (13 instead of 8 hydroxylysine residues, expressed per 1000 amino acids).

Effect of diphosphonates on the hydroxylation of proline

Contrary to the effect of dichloromethanediphosphonate on the hydroxylation of lysine, no evidence was found of either diphosphonate altering the hydroxylation of proline (Table 3).

Effect of diphosphonates on intermolecular cross-links

A typical elution pattern of NaB^3H_4-reduced cross-links of collagen from rat calvaria is illustrated in Fig. 2. The elution profiles obtained from acid-hydrolysed collagen of control (I), dichloromethanediphosphonate-treated (II) and 1-hydroxyethane-1,1-diphosphonate-treated (III) rat calvaria demonstrate that, in all three cases, dihydroxylysinonorleucine (i) and hydroxylysinonorleucine (ii)



represent the two major cross-links, whereas lysinonorleucine (iii) and histidinohydroxymerodesmosine (iv) were identified as being, by their quantity, minor cross-links. Moreover, Fig. 2(*b*) illustrates that the peak marked as dihydroxylysinonorleucine appears to be larger in area from collagen of dichloromethanediphosphonate-treated rats than those of 1-hydroxyethane-1,1-diphosphonate-treated ones or from collagen of rats that were not treated at all (Fig. 2*a*). The quantitative data of the two major cross-links are summarized in Table 4. Although collagen from all dichloromethanediphosphonate-exposed tissues examined showed an increase in dihydroxylysinonorleucine residues, a condensation product of two hydroxylysine residues, a concomitant decrease in hydroxylysinonorleucine residues, the condensation product of one lysine and one hydroxylysine residue, was noticed. These data clearly demonstrate the close relationship between the number of hydroxylysine residues present in collagen (Table 2) and the number of the dihydroxylysinonorleucine cross-links formed (Table 4). 1-Hydroxyethane-1,1-diphosphonate did not induce qualitative changes in the formation of cross-links. This was to be expected in view of the findings above (Table 2), where it was shown that this diphosphonate did not display a stimulatory effect on the conversion of lysine into hydroxylysine. Finally, as shown in Table 4, a maximum in the number of dihydroxylysinonorleucine residues was obtained already with a dose of 5 mg of P · (kg of body wt.)$^{-1}$. An increase in dichloromethanediphosphonate up to a dose of 10 mg of P · kg^{-1} did not result in a further increase of dihydroxylysinonorleucine.

Discussion

The results of the present study provide evidence

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of CNBr peptides of collagen from tibial heads (a), tibial shafts (b) and calvaria (c) of rats treated with dichloromethanediphosphonate*

About 100 μg of each digest was run in 15% (w/v) acrylamide gel (100 mm × 5 mm) for 5 h at constant voltage of 200 V. Gels were stained with Coomassie Brilliant Blue R and scanned at 550 nm. The various CNBr peptides of type-I and type-II collagen of the rat were identified as described by Scott & Veis (1976). Arrows indicate the location of the collagen-type-specific CNBr peptides. Peptide α1(II)CB10 specific for cartilage type-II collagen does not appear on CNBr profile of calvaria collagen (*c*) and hence is devoid of type-II collagen. CNBr digests of collagen from tibial shafts and tibial heads contain both CNBr peptides.

Table 3. *Effect of diphosphonates on the hydroxylation of proline*

The results given below are ratios of radiolabelled hydroxyproline to proline and represent the means ± S.E.M. for three separate experiments (comprised of a pool of eight to ten rats). The diphosphonates were administered *in vivo* as described in the Experimental section. After treatment, the organs were explanted and cultured *in vitro* in the presence of [^{3}H]proline.

	Radiolabelled-hydroxyproline/proline ratio		
Tissue ...	Calvaria	Tibial head	Tibial shafts
Control	0.77 ± 0.02	0.73 ± 0.04	0.76 ± 0.06
Dichloromethanediphosphonate-treated (10 mg of P·kg^{-1})	0.76 ± 0.01	0.75 ± 0.03	0.76 ± 0.02
1-Hydroxyethane-1,1-diphosphonate-treated (10 mg of P·kg^{-1})	0.77 ± 0.04	0.75 ± 0.01	0.76 ± 0.04

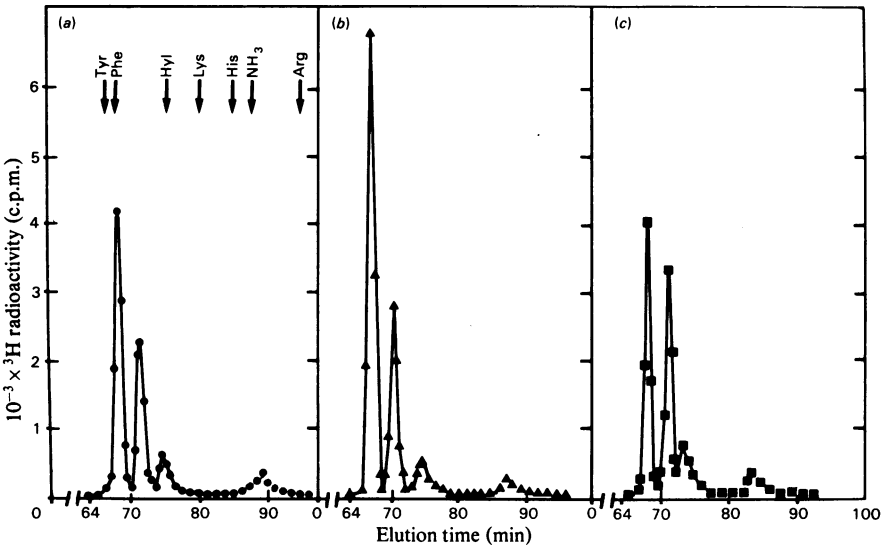


Fig. 2. *Elution pattern of NaB^{3}H$_4$-reduced cross-links of calvaria collagen from rats treated or not treated with diphosphonate*

Preparation of collagen, treatment with NaB^{3}H$_4$ and separation of the individual cross-links were performed as described in the Experimental section. (*a*), (*b*) and (*c*) depict the elution profile of reduced cross-links of calvaria collagen from controls, dichloromethanediphosphonate-treated and 1-hydroxyethane-1,1-diphosphonate-treated rats respectively. The individual cross-links [(i) dihydroxylysinonorleucine, (ii) hydroxylysinonorleucine, (iii) lysinonorleucine and (iv) histidinohydroxymerodesmosine] were identified as described by Kao & Leslie (1979).

that dichloromethanediphosphonate, but not 1-hydroxyethane-1,1-diphosphonate, when administered to the young growing rat, stimulates the formation of hydroxylysine residues found on the α-chains of bone, cartilage and skin collagen. The effect on skin collagen constitutes an unexpected finding, since in previous studies it was found that diphosphonates act only on bone and cartilage. This specific action was explained by the fact that diphosphonates are 'bone seekers'; once administered, they become bound to the calcium apatite of the skeleton and thus are available to other tissues in rather small amounts (Jung *et al.*, 1973). The data obtained for skin, however, seem to indicate that other tissues can respond as well. The fact that the stimulatory effect on the hydroxylation of lysine was not paralleled by an increased number of hydroxyproline residues suggests that the observed effects are specific rather than constitute a general phenomenon on hydroxylation.

Furthermore, on the basis of CNBr digestion and subsequent analysis of the resulting collagen CNBr fragments by polyacrylamide-gel electrophoresis, it can safely be stated that the augmented hydroxylysine content is not due to tissue heterogeneity (Fig. 1), but to the diphosphonate.

Several studies performed in the past have shown that the number of existing hydroxylysine residues found in collagen may vary not only between different tissues but in the same tissue of animals of different age as well (Miller *et al.*, 1967; Barnes *et al.*, 1971). The latter phenomenon is explained by

Table 4. *Effect in vivo of diphosphonates on collagen intermolecular-cross-link formation*

Results refer to the two major cross-links, dihydroxylysinonorleucine (DHLNL) and hydroxylysinonorleucine (HLNL), and are expressed as percentage of the sum of all NaB^3H_4-reduced cross-links determined, which includes lysinonorleucine and histidinohydroxymerodesmosine. The determination of cross-links was performed as described in the Experimental section.

Tissue		Percentage of total cross-links: DHLNL	Percentage of total cross-links: HLNL	DHLNL/HLNL
Calvaria	Control	51.3	37.8	1.36
	Dichloromethanediphosphonate-treated			
	5mg of P·kg^{-1}	58.6	32.7	1.79
	10mg of P·kg^{-1}	61.7	30.2	2.04
	1-Hydroxyethane-1,1-diphosphonate-treated			
	5mg of P·kg^{-1}	51.4	38.7	1.33
	10mg of P·kg^{-1}	50.5	38.9	1.30
Cartilage	Control	64.9	18.9	3.43
	Dichloromethanediphosphonate-treated			
	5mg of P·kg^{-1}	77.0	15.3	5.03
	10mg of P·kg^{-1}	74.9	14.2	5.27
	1-Hydroxyethane-1,1-diphosphonate-treated			
	5mg of P·kg^{-1}	72.0	21.7	3.32
	10mg of P·kg^{-1}	65.2	22.3	2.92
Tibia				
	Control	60.2	35.9	1.68
	Dichloromethanediphosphonate-treated (10mg of P·kg^{-1})	72.4	19.3	3.75
	1-Hydroxyethane-1,1-diphosphonate-treated (10mg of P·kg^{-1})	56.8	36.5	1.56

the fact that although with increasing age the synthesis of collagen decreases, the activity of lysine hydroxylase (Anttinen *et al.*, 1973) and also proline hydroxylase (Cardinale & Udenfriend, 1974; Kivirikko, 1970) likewise decreases. Whereas the hydroxylation of proline residues appears to be a very efficient process, meaning that virtually all eligible proline residues become hydroxylated (Hulmes *et al.*, 1973), the hydroxylation of lysine proceeds less efficiently. This suggests that the number of hydroxylated lysine residues is dependent on the amount and/or activity of lysine hydroxylase. Since it is known that foetal collagen contains a larger amount of hydroxylysine residues (Miller *et al.*, 1967; Barnes *et al.*, 1974), it is conceivable that the increased collagen, synthesized under the influence of dichloromethanediphosphonate [see the preceding paper (Guenther *et al.*, 1981], constitutes a 'foetal' type of collagen.

Shuttleworth & Forrest (1975) have shown that, in dermal scar tissue, where collagen synthesis is high, a concomitant increase in hydroxylysine content was evident.

Some years ago an increase in the number of hydroxylysine residues was described in bone collagen of chicks that had been raised on a vitamin D-deficient diet (Toole *et al.*, 1972; Barnes *et al.*, 1973*a*,*b*; Mechanic *et al.*, 1975). Recently Dickson *et al.* (1979) were able to find an apparent inverse correlation between plasma calcium concentration and the extent of lysine hydroxylation. The probability that the action of dichloromethanediphosphonate on the hydroxylation of lysine residues may have been mediated by a decrease in plasma calcium concentration can be dismissed. Past studies have shown that dichloromethanediphosphonate given *in vivo* to either chicks or rats did not alter calcaemia to any significant extent (Bonjour *et al.*, 1973*a*). Moreover, since it was also found that the diphosphonate did not interfere with the metabolism of cholecalciferol (vitamin D_3) (Bonjour *et al.*, 1973*b*), the effect on hydroxylation could not have been caused by a lack of active cholecalciferol metabolites.

The participation of lysine and hydroxylysine in collagen cross-link formation ultimately must produce changes in the type of cross-link formed, if the ratio of lysine to hydroxylysine becomes changed. The effect of dichloromethanediphosphonate, as well as that of 1-hydroxyethane-1,1-diphosphonate, on cross-links (shown in Table 4) supports this concept. The cross-link dihydroxylysinonorleucine appears increased in collagen of dichloromethanediphosphonate-treated animals, whereas, at the same time, the amount of hydroxylysinonorleucine was found to have decreased.

The appearance of hydroxylated lysine residues in the cross-link region renders increased stability to collage intermolecular cross-links. It has been proposed that cross-links made up of lysine residues only are less stable than those formed of hydroxylysine residues (Robins *et al.*, 1973; Boucek *et al.*, 1979). This may be in part due to the Amadorni rearrangement, which favours the stabilization of the acid-labile Schiff base, an intermediate form in the sequence of cross-link formation (Eyre & Glimcher, 1973). The decrease in solubility of collagen from dichloromethanediphosphonate-treated rats in acetic

acid (Table 1) appears to be in accordance with this hypothesis.

It is known that dichloromethanediphosphonate blocks bone resorption (Fleisch *et al.*, 1969; Russell *et al.*, 1970). In newborn rats, dichloromethanediphosphonate causes an impairment of normal bone remodelling and produces a skeleton that resembles that of the 'grey lethal' strain of congenitally osteopetrotic mice (Reynolds *et al.*, 1973). Studies on osteopetrotic bone (Banes *et al.*, 1978) revealed an increase in the ratio of dihydroxylysinonorleucine to hydroxylysinonorleucine. In Paget's disease (Meunier *et al.*, 1979) as well as in osteolytic-tumoral-bone disease (Siris *et al.*, 1980), dichloromethanediphosphonate is used as a treatment to decrease bone resorption. Recent studies have provided evidence that the number and/or quality of existing cross-links determine to a great extent the susceptibility of collagen to collagenase breakdown (Vater *et al.*, 1979; Harris & McCroskery, 1974; Boucek *et al.*, 1979). It is possible that the synthesis of a collagen that exhibits increased resistance to collagen catabolism, because of altered cross-links, may contribute to this inhibition of bone destruction induced by the diphosphonates.

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References

Anttinen, H., Orava, S., Ryhänen, L. & Kivirikko, K. I. (1973) *Clin. Chim. Acta* **47**, 289–294

Banes, A. J., Bernstein, P. H., Smith, R. E. & Mechanic, G. L. (1978) *Biochem. Biophys. Res. Commun.* **81**, 1390–1397

Barnes, M. J., Constable, B. J., Morton, L. F. & Kodicek, E. (1971) *Biochem. J.* **125**, 433–437

Barnes, M. J., Constable, B. J., Morton, L. F. & Kodicek, E. (1973*a*) *Biochem. J.* **132**, 113–115

Barnes, M. J., Constable, B. J., Morton, L. F. & Kodicek, E. (1973*b*) *Biochim. Biophys. Acta* **328**, 373–382

Barnes, M. J., Constable, B. J., Morton, L. F. & Royce, P. M. (1974) *Biochem. J.* **139**, 461–468

Berg, R. A. & Prockop, D. J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 115–120

Bonjour, J.-P., Fleisch, H. & Copp, D. H. (1973*a*) *Proc. Soc. Exp. Biol. Med.* **143**, 404–407

Bonjour, J.-P., LeLuca, H. F., Fleisch, H., Trechsel, U., Matejowec, L. A. & Omdahl, J. L. (1973*b*) *Eur. J. Clin. Invest.* **3**, 44–48

Boucek, R. J., Noble, N. L. & Gunja-Smith, Z. (1979) *Biochem. J.* **177**, 853–860

Cardinale, G. J. & Udenfried, S. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol.* **41**, 245–300

Dickson, I. R., Eyre, D. R. & Kodicek, E. (1979) *Biochim. Biophys. Acta* **588**, 169–173

Eyre, D. R. & Glimcher, M. J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 663–671

Fleisch, H., Russell, R. G. G. & Francis, M. D. (1969) *Science* **165**, 1262–1264

Guenther, H. L., Croissant, R. D., Schonfeld, S. E. & Slavkin, H. C. (1977) *Biochem. J.* **163**, 591–603

Guenther, H. L., Guenther, H. E. & Fleisch, H. (1981) *Biochem. J.* **196**, 293–301

Harris, E. D., Jr. & McCroskery, P. A. (1974) *N. Engl. J. Med.* **290**, 1–6

Hulmes, D. J. S., Miller, A., Parry, D. A. D., Piez, K. A. & Woodhead-Galloway, J. (1973) *J. Mol. Biol.* **79**, 137–148

Jimenez, S., Harsch, M. & Rosenbloom, J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 106–114

Jung, A., Bisaz, S. & Fleisch, H. (1973) *Calcif. Tissue Res.* **11**, 269–280

Kao, K. Y. T. & Leslie, J. G. (1979) *Biochim. Biophys. Acta* **580**, 366–371

Kivirikko, K. I. (1970) *Int. Rev. Connect. Tissue Res.* **5**, 93–163

Lane, J. M. & Miller, E. J. (1969) *Biochemistry* **8**, 2134–2139

Mechanic, G. L., Toverud, S. U., Ramp, W. K. & Gonnermann, W. A. (1975) *Biochim. Biophys. Acta* **393**, 419–425

Meunier, P. J., Chapuy, M. C., Alexandre, C., Bressot, C., Edouard, C., Vignon, E., Mathieu, L. & Trechsel, U. (1979) *Lancet* **ii**, 489–492

Miller, E. J. (1973) *Clin. Orthop.* **92**, 260–280

Miller, E. J. & Robertson, P. B. (1973) *Biochem. Biophys. Res. Commun.* **54**, 432–440

Miller, E. J., Martin, G. R., Piez, K. A. & Powers, M. J. (1967) *J. Biol. Chem.* **242**, 5481–5489

Miller, E. J., Lane, J. M. & Piez, K. A. (1969) *Biochemistry* **8**, 30–39

Nimni, M. E. (1974) *Semin. Arthritis Rheum.* **4**, 95–150

Reynolds, J. J., Murphys, H., Mühlbauer, R. C., Morgan, D. B. & Fleisch, H. (1973) *Calcif. Tissue Res.* **12**, 59–71

Robins, S. P., Shimokomaki, M. & Bailey, A. J. (1973) *Biochem. J.* **131**, 771–780

Roth, M. (1976) *J. Clin. Chem. Clin. Biochem.* **14**, 361–364

Russell, R. G. G., Mühlbauer, R. C., Bisaz, S., Williams, D. A. & Fleisch, H. (1970) *Calcif. Tissue Res.* **6**, 183–196

Scott, P. G. & Veis, A. (1976) *Connect. Tissue Res.* **4**, 107–116

Shuttleworth, C. A. & Forrest, L. (1975) *Eur. J. Biochem.* **55**, 391–395

Siris, E. S., Sherman, W. H., Baquiron, D. C., Schlatterer, J. P., Osserman, E. F. & Canfield, R. E. (1980) *N. Engl. J. Med.* **302**, 310–315

Stegemann, H. & Stalder, K. H. (1967) *Clin. Chim. Acta* **18**, 267–273

Toole, B. P., Kang, A. H., Trelstad, R. L. & Gross, J. (1972) *Biochem. J.* **127**, 715–720

Vater, C. A., Harris, E. D., Jr. & Siegel, R. C. (1979) *Biochem. J.* **181**, 639–645