Biochimica et Biophysica Acta, 429 (1976) 429—438

BBA 67776

THE EFFECT OF SEVERAL DIPHOSPHONATES ON ACID PHOSPHOHYDROLASES AND OTHER LYSOSOMAL ENZYMES

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(Received September 1st, 1975)
(Revised manuscript received December 9th, 1975)

Summary

Diphosphonates are known to inhibit bone resorption in tissue culture and in experimental animals. This effect may be due to their ability to inhibit the dissolution of hydroxyapatite crystals, but other mechanisms may be important. Since lysosomal enzymes have been implicated in the process of bone resorption, we have examined the effect of several phosphonates and of a polyphosphate ($P_{20,i}$) on lysosomal hydrolases derived from rat liver and rat bone. Dichloromethylene diphosphonate strongly inhibited acid β-glycerophosphatase (EC 3.1.3.2) and acid *p*-nitrophenyl phosphatase (EC 3.1.3.2) and to a lesser degree (in descending order) acid pyrophosphatase (EC 3.1.3.-), arylsulfatase A (EC 3.1.6.1), deoxyribonuclease II(EC 3.1.4.6) and phosphoprotein phosphatase (EC 3.1.3.16) of rat liver. Inhibition of acid *p*-nitrophenyl phosphatase and arylsulfatase A was competitive. Ethane-1-hydroxy-1,1-diphosphonate did not inhibit any of these enzymes, except at high concentrations. Neither dichloromethylene diphosphonate nor ethane-1-hydroxy-1,1-diphosphonate had any effect on β-glucuronidase (EC 3.2.1.31), arylesterase (EC 3.1.1.2) and cathepsin D (EC 3.4.23.5). Of several other phosphonates tested only undec-10-ene-1-hydroxy-1,1-diphosphonic acid inhibited acid *p*-nitrophenyl phosphatase strongly, the polyphosphate ($P_{20,i}$) had little effect. Acid *p*-nitrophenyl phosphatase in rat calvaria extract behaved in the same way as the liver enzyme and was also strongly inhibited by dichloromethylene diphosphonate, but not by ethane-1-hydroxy-1,1-diphosphonate.

It is suggested that the inhibition of bone resorption by dichloromethylene diphosphonate might be due in part to a direct effect of this diphosphonate on lysosomal hydrolases.

Introduction

Various diphosphonates, characterised by P-C-P bonds, inhibit bone resorption in tissue culture and in experimental animals [1—3]. These effects may be due to the ability of such compounds to inhibit the dissolution of hydroxyapatite crystals [2], but it is possible that other mechanisms might be important. There is evidence that lysosomal enzymes might be involved in bone resorption [4]. Thus, when bone resorption is stimulated by parathyroid hormone in tissue culture, an increase occurs in the production and release of lactate and of lysosomal enzymes [5,6]. This increase in lactate production and in lysosomal enzymes is blocked by dichloromethylene diphosphonate [7].

It is possible that diphosphonates may not only inhibit the system but also the activity of certain lysosomal enzymes, particularly acid phosphatase, because diphosphonates are related to pyrophosphate, and pyrophosphate is a substrate for acid phosphatase. We have therefore studied the direct effect of several diphosphonates on lysosomal hydrolases in vitro. In particular we have compared dichloromethylene diphosphonate, the diphosphonate which strongly inhibits bone resorption, with ethane-1-hydroxy-1,1-diphosphonate, a diphosphonate which inhibits bone resorption less strongly [2,8]. The majority of studies were done with enzymes obtained from the light mitochondrial fraction of rat liver, because liver fractionation is more easy than bone fractionation, but confirmatory experiments were done on acid phosphatase from bone.

Materials

Animals. Young female Wistar rats weighting 180—245 g and from our own breeding stock were used for the preparation of the light mitochondrial fraction of liver. 1—2-day-old Wistar rats were used for the preparation of an extract of calvaria.

TABLE I

LIST OF THE COMPOUNDS STUDIED

For structural formulae, see ref. 9.

UHDP is $CH_2 = CH(CH_2)_8-C(PO_3H_2)_2-OH$.

Name	Abbreviation
Inorganic polyphosphate (n = 20)	P_{20},i
n-Pentanemonophosphonic acid	PMP
Ethane-1,2-diphosphonic acid	E-1,2-DP
Methylenediphosphonic acid	MDP
Methylenehydroxydiphosphonic acid	MHDP
Dichloromethylenediphosphonic acid	Cl_2MDP
Ethane-1-amino-1,1-diphosphonic acid	EADP
Ethane-1-hydroxy-1,1-diphosphonic acid	EHDP
Undec-10-ene-1-hydroxy-1,1-diphosphonic acid	UHDP
Ethane-1-hydroxy-1,1,2-triphosphonic acid	E-1-HTP
Propane-1,1,3,3-tetraphosphonic acid	PTeP
A polyester chain condensate of EHDP with acetic anhydride	Condensate I

Chemicals. DNA (from calf thymus; mol. wt. 1 200 000) and nitrocatechol (1,2-dihydroxy-4-nitrobenzene) and bovine serum albumin were obtained from Fluka AG, Buchs, Switzerland; Dowex 50-WX4, 100—200 mesh, pract. from Serva, Heidelberg, W. Germany. The polyphosphate ($n = 20$) was chromatographically pure and was a gift from D.G. Berg, Rochester, N.Y., U.S.A. The phosphonates (Table I) [9] were from the Procter and Gamble Comp., Cincinnati, Ohio, U.S.A. All other reagents were of analytical grade from E. Merck AG, Darmstadt, W. Germany.

Methods

Preparation of lysosomal enzymes of rat liver

The rat livers were fractionated according to the method of de Duve et al. [10]. Table II shows the distribution of protein and acid phosphatase onto the different fractions. The light mitochondrial fraction (containing the lysosomes) was lysed by adding 1 ml distilled water per g of original liver weight, followed by freezing and thawing five times. After centrifugation at 37 000 × g_{av} for 20 min (100 000 × g_{av} for 30 min when the supernatant was used to measure acid phosphatase with *p*-nitrophenyl phosphate as substrate), the supernatant was taken and stored frozen, before use as the enzyme source. Esterase activity is known to occur on lysosomal membranes (pH optimum 5.0) as well as in the microsomal fraction (pH optimum 8.5) [11]. In this study both esterases were assayed in homogenates from which the nuclei and the heavy mitochondrial fraction had been removed.

Extract of rat calvaria

The calvaria were obtained from 50 1 to 2-day-old rats. They were washed in cold 0.9% NaCl, 10 mM Tris · HCl, pH 7.4, and homogenized in a 1.0 ml glass homogenizer (0.8 ml distilled water per five calvaria). It was centrifuged at 600 × *g* for 15 min and the pellet extracted twice. The collected supernatants were frozen and thawed five times, centrifuged at 100 000 × g_{av} for 30 min and the

TABLE II

FRACTIONAL DISTRIBUTION OF PROTEIN AND ACID PHOSPHATASE

Acid phosphatase was measured using *p*-nitrophenyl phosphate as substrate. The enzyme was preincubated for 15 min at 37°C and 0.1% Triton X-100 was used as releasing agent. The mean ±1 S.E. of four experiments is given. E, cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant. Relative specific activity means: percent acid phosphatase/percent protein.

Fraction	Percentage of		Relative specific activity
	Protein	Acid phosphatase	
E + N	100	100	1.0
N	24.5 ± 1.4	18.6 ± 1.6	0.76 ± 0.06
M	14.1 ± 0.6	25.0 ± 1.4	1.78 ± 0.13
L	3.3 ± 0.5	17.7 ± 0.2	5.78 ± 0.88
P	17.6 ± 2.0	18.7 ± 1.0	1.09 ± 0.08
S	36.9 ± 1.3	23.1 ± 0.8	0.63 ± 0.003
Recovery	96.4 ± 2.1	103.2 ± 0.5	

supernatant dialysed overnight against 10 mM acetate buffer, pH 5.0. Precipitated protein was removed by centrifugation. The 10 ml supernatant obtained was diluted to 20 ml (0.05 M acetate buffer, pH 5.0) and incubated for 20 min at 37°C to destroy glucose-6-phosphatase. This extract was used to make the Lineweaver-Burk analysis in Fig. 4.

Enzyme assays

Acid phosphatase (EC 3.1.3.2). When *p*-nitrophenyl phosphate was used as substrate, 50—100 µl enzyme were incubated in 1.0 ml final volume, 0.2 M acetate buffer, pH 5.0, at 37°C for 15—30 min [12]. To destroy glucose-6-phosphatase, the enzyme solution was preincubated in acetate buffer, pH 5.0, at 37°C for 15 min. Absorbance was measured at 405 nm.

When β-glycerophosphate was used as substrate, 20 µl enzyme were incubated in a medium containing 50 mM β-glycerophosphate in 50 mM sodium acetate buffer at pH 5.0. After 30 min the reaction was stopped by adding 1 ml 10% (w/v) trichloroacetic acid and the release of phosphate was determined by the Elon procedure [12].

Acid pyrophosphatase (EC 3.1.3.-). 20 µl enzyme were incubated with 0.5 ml medium containing 1 mM tetrasodium pyrophosphate (PP_i) and 5 mM ascorbic acid in 0.1 M acetate buffer at pH 5.2. After 30 min the reaction was stopped by adding 10% (w/v) trichloroacetic acid containing 5 mM $CuSO_4$; the phosphate released was determined by the method of Wöltgens and Ahsmann [13].

Phosphoprotein phosphatase (EC 3.1.3.16). 100 µl enzyme were incubated with 1 ml of 0.1 M sodium acetate buffer at pH 5.8 containing 5 mM ascorbic acid and casein (Hammarsten) equivalent to 7 µmol total P_i and dissolved according to Revel and Racker [14]. After 60 min the reaction was stopped by adding 1 ml 20% (w/v) acetic acid. About 250 mg Dowex 50-WX4 was added and mixed on a Vortex mixer. After centrifugation 1 ml of the supernatant was added to 1 ml water and the P_i released determined by the Elon procedure [12].

Deoxyribonuclease II (EC 3.1.4.6). This was determined by the method of Barrett [12]. 40 µl enzyme were incubated with 1 ml of incubation medium for 30 min.

Arylsulfatase A (EC 3.1.6.1). The method described by Barrett [12] was used. 75 µl enzyme were incubated for 30 min with 0.6 ml incubation medium containing 2-hydroxy-5-nitrophenyl sulfate (nitrocatechol sulfate).

β-Glucuronidase (EC 3.2.1.31). 10 µl enzyme were incubated in 0.5 ml of 0.075 M acetate buffer at pH 5.2 containing 1.25 mM 4-nitrophenyl-β-D-glucopyranosiduronic acid. After 10 min the reaction was stopped by adding 2 ml of 0.1 M NaOH and the absorbance was measured at 405 nm.

Cathepsin D (EC 3.4.23.5). This enzyme was determined by the method of Barrett [12]. Haemoglobin was used as substrate and the liberated peptides measured with the Folin Lowry reaction. 20 µl enzyme were incubated with 0.5 ml of substrate solution at 45°C for 30 min.

Arylesterase (EC 3.1.1.2). This enzyme was measured at pH 5.0 and 8.5: 20 µmol 2-naphthylacetate dissolved in 2 ml (1 ml for pH 8.5) acetone were rapidly mixed with 18 ml 0.1 M citrate/phosphate buffer, pH 5.0, or 19 ml 0.1 M barbital buffer at pH 8.5. Aliquots of 0.5 ml were taken as incubation medium.

10 µl of homogenate (1 : 15 diluted with water for pH 5.0) or 20 µl of homogenate (1 : 100 diluted with water for pH 8.5) were incubated for 15 min. The reaction was stopped by adding 0.5 ml ice-cold ethanol. After cooling for 5 min it was centrifuged. 0.5 ml diazo blue BB (4 mg/ml) were added to 0.5 ml supernatant. After 1 min, 1 ml of 5 mM KOH ethanol was added and the absorbance measured at 530 nm after 10 min [11].

All enzyme reactions except for cathepsin D were done at 37°C. Changes of the usual substrate concentration and of the usual incubation time are described in the text. In all cases the enzyme reaction was shown to be linearly proportional to time and the amount of enzyme added.

Protein determination

This was determined according to Lowry et al. [15] using bovine serum albumin as standard.

Results

The effect of ethane-1-hydroxy-1,1-diphosphonate and dichloromethylene diphosphonate on enzymes from rat liver lysosomes

Fig. 1 shows the effect of ethane-1-hydroxy-1,1-diphosphonate and dichloro-

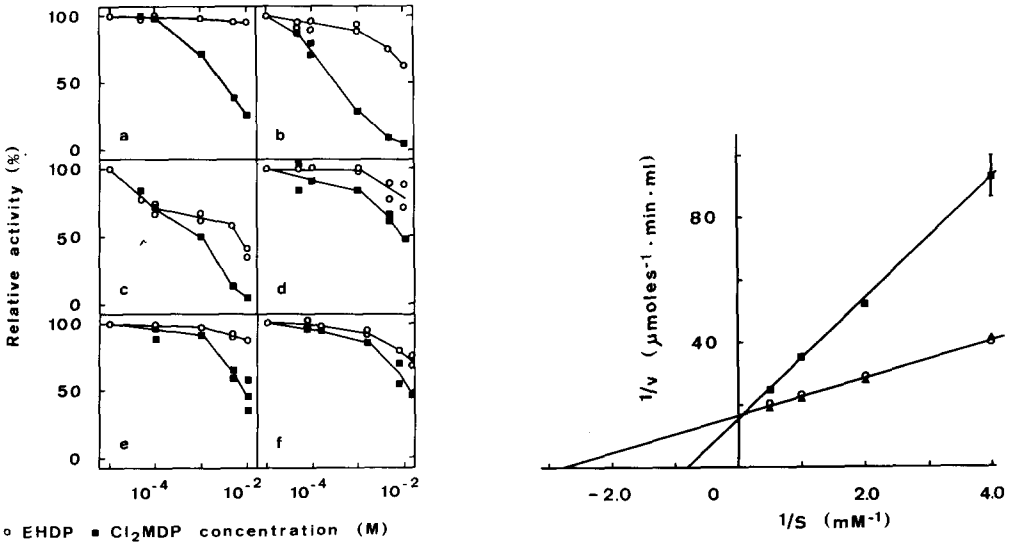

Fig. 1. The effect of ethane-1-hydroxy-1,1-diphosphonate and dichloromethylene diphosphonate on hydrolases of liver lysosomes. The relative activity is expressed as a percentage of the values found in the absence of diphosphonates. a, acid *p*-nitrophenyl phosphatase; b, acid β-glycerophosphatase; c, acid pyrophosphatase; d, phosphoprotein phosphatase; e, DNAase II; f, arylsulfatase A. ○, ethane-1-hydroxy-1,1-diphosphonate; ■, dichloromethylene diphosphonate. Each experiment was done two or three times. Only one point is shown when the values were indistinguishable graphically.

Fig. 2. Lineweaver-Burk plot for lysosomal acid phosphatase of rat liver. The enzyme solution had been preincubated in 0.05 M acetate buffer, pH 5.0, at 37°C for 15 min. The activity was determined by incubating 50 µl enzyme solution (0.95 mg protein/ml) in 0.4 M KCl, 0.1 M acetate buffer, pH 5.0, using 0.25—2.0 mM *p*-nitrophenyl phosphate as substrate in the absence of diphosphonate (○), at 0.5 mM ethane-1-hydroxy-1,1-diphosphonate (▲) and at 0.5 mM dichloromethylene diphosphonate (■). *v*, velocity; *S*, substrate concentration. The experiment was done three times. The mean value ± 2 S.E. is given. 2 S.E. smaller than 1.0 µmol^{-1} · min · ml are not drawn.

methylene diphosphonate on various lysosomal enzymes. Dichloromethylene diphosphonate inhibited the acid phosphatase when *p*-nitrophenyl phosphate or β-glycerophosphate was used as substrate. In the case of β-glycerophosphate inhibition was detected at 0.05 mM dichloromethylene diphosphonate. Ethane-1-hydroxy-1,1-diphosphonate had no effect on acid phosphatase except at high concentrations. The effects of dichloromethylene diphosphonate and ethane-1-hydroxy-1,1-diphosphonate were studied using various concentrations of *p*-nitrophenyl phosphate as substrate. The results are shown in Fig. 2 as a Lineweaver-Burk plot. Ethane-1-hydroxy-1,1-diphosphonate showed no significant inhibition whereas dichloromethylene diphosphonate inhibited competitively (K_m for *p*-nitrophenyl phosphate, 0.37 mM; K_i for dichloromethylene diphosphonate, 0.20 mM).

When PP_i was used as substrate both dichloromethylene diphosphonate and ethane-1-hydroxy-1,1-diphosphonate inhibited the enzyme to about the same extent at 0.05 mM, although at higher concentrations dichloromethylene diphosphonate was the stronger inhibitor of the two (Fig. 1).

Dichloromethylene diphosphonate and ethane-1-hydroxy-1,1-diphosphonate inhibited deoxyribonuclease II activity and the phosphoprotein phosphatase with casein as substrate only at concentrations above 1 mM; with dichloromethylene diphosphonate having the stronger effect (Fig. 1). In order to see whether these inhibitory effects might be more obvious at lower concentrations of substrate, the assays were repeated using lower concentrations of casein and DNA. As seen from Table IIIa and IIIb, 0.1 or 1 mM ethane-1-hydroxy-1,1-diphosphonate had little or no inhibitory effect even when the substrate concentrations were lowered 10—12 times. Dichloromethylene diphosphonate in-

TABLE III

THE EFFECT OF ETHANE-1-HYDROXY-1,1-DIPHOSPHONATE AND DICHLOROMETHYLENE DIPHOSPHONATE ON PHOSPHOPROTEIN PHOSPHATASE AND DEOXYRIBONUCLEASE II AT DIFFERENT CASEIN AND DNA CONCENTRATIONS

The activity (mean ± S.E.) is expressed as a percentage of the values found at different substrate concentrations in the absence of diphosphonates. Number of experiments shown in parentheses.

(a) Casein (μM total P_i)	Activity as percent of control	
	1 mM Ethane-1-hydroxy-1, 1-diphosphonate	1 mM Dichloromethylene diphosphonate
0.6	98, 101 (2)	84, 85 (2)
0.2	96.5 ± 2.9 (4)	80.0 ± 3.6 (6)
0.05	94.8 ± 3.8 (4)	77.7 ± 4.1 (6)

(b) DNA (mg/ml)	Activity as percent of control			
	Ethane-1-hydroxy-1,1-diphosphonate		Dichloromethylene diphosphonate	
	0.1 mM	1.0 mM	0.1 mM	1.0 mM
1	98, 99 (2)	95, 98 (2)	88, 97 (2)	91.9 ± 2.9 (3)
0.1	98.2 ± 2.6 (6)	92.0 ± 2.7 (6)	81.2 ± 5.4 (6)	82.3 ± 3.1 (6)

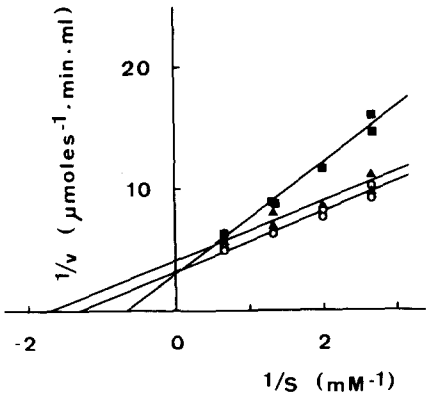

Fig. 3. Lineweaver-Burk plot for liver arylsulfatase A. The enzyme was incubated for 20 min in 0.6 ml medium containing 0.375—1.5 mM nitrocatechol sulfate as described under Methods. ○, no diphosphonates; ▲, 0.78 mM ethane-1-hydroxy-1,1-diphosphonate; ■, 0.78 mM dichloromethylene diphosphonate. v, velocity; S, substrate concentration. Each experiment was done twice and the individual values are given. If they coincided, only one point is drawn.

hibited to a somewhat greater degree at the lower substrate concentrations.

Dichloromethylene diphosphonate also inhibited arylsulfatase A, again to a greater extent than ethane-1-hydroxy-1,1-diphosphonate. Fig. 3 shows a Lineweaver-Burk plot derived from assays at different substrate concentrations. Dichloromethylene diphosphonate inhibited competitively (K_m for nitrocatechol

TABLE IV

EFFECT OF POLYPHOSPHATE AND PHOSPHONATES ON LYSOSOMAL ACID PHOSPHATASE OF RAT LIVER

2.0 or 0.5 mM *p*-nitrophenyl phosphate was used as substrate. The activities are expressed as percentage of the control obtained when no polyphosphate or phosphonate was added. The mean ± S.E. of three experiments is given.

Phosphonates *	Substrate concentration			
	2.0 mM		0.5 mM	
	Phosphonate concentration			
	0.1 mM	1.0 mM	0.1 mM	1.0 mM
Cl_2MDP	82.7 ± 1.1	37.0 ± 0.4	55.6 ± 0.2	13.7 ± 0.2
UHDP	83.2 ± 0.7	45.9 ± 0.2	56.3 ± 0.5	17.8 ± 0.1
MHDP	97.8 ± 0.9	94.0 ± 0.8	97.4 ± 0.4	80.9 ± 0.5
$P_{20,i}$ **	97.0 ± 0.3	95.5 ± 1.0	96.6 ± 0.8	88.2 ± 0.5
Condensate I	98.4 ± 1.2	96.9 ± 0.3	97.8 ± 0.8	86.6 ± 0.3
EHDP	98.4 ± 0.8	97.2 ± 0.3	98.0 ± 0.9	88.2 ± 0.4
E-1-HTP	98.8 ± 0.5	98.3 ± 0.1	98.7 ± 0.8	88.3 ± 0.8
EADP	99.7 ± 0.7	98.4 ± 0.8	98.4 ± 0.7	94.2 ± 1.0
PTeP	98.1 ± 0.5	98.6 ± 0.7	99.1 ± 0.8	95.6 ± 0.5
MDP	99.1 ± 0.9	99.9 ± 0.4	100.0 ± 0.9	96.8 ± 0.5
E-1,2-DP	99.0 ± 0.8	100.2 ± 0.6	99.3 ± 0.7	98.6 ± 0.5
PMP	98.4 ± 0.8	99.9 ± 0.2	99.1 ± 0.9	97.7 ± 0.7

* Abbreviations, see Table I.

** The polyphosphate $P_{20,i}$ was added at 0.01 and 0.1 mM instead of 0.1 and 1.0 mM. This corresponds to phosphorus concentrations of 0.1 and 1.0 mM diphosphonate.

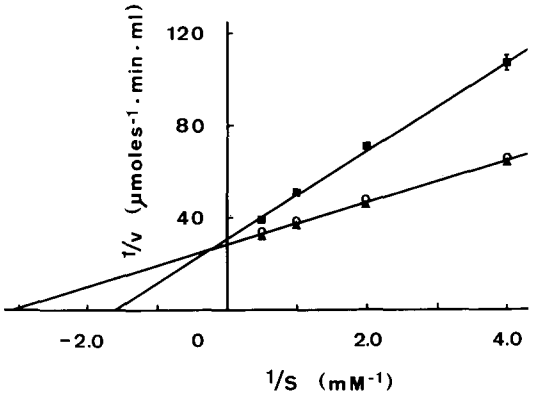

Fig. 4. Lineweaver-Burk plot for acid phosphatase from rat calvaria extract. 50 μl of the extract (0.265 mg protein/ml) obtained as described under Methods, were incubated for 20 min at 37°C in 1 ml 0.1 M acetate buffer, pH 5.0, 0.4 M KCl and 0.25—2.0 mM *p*-nitrophenyl phosphate in the absence of diphosphonates (○) and in the presence of 0.5 mM ethane-1-hydroxy-1,1-diphosphonate (▲) and 0.5 mM dichloromethylene diphosphonate (■). *v*, velocity; *S*, substrate concentration. The experiment was done three times. The mean value ± 2 S.E. is given. 2 S.E. smaller than 1.2 $\mu mol^{-1} \cdot min \cdot ml$ are not drawn.

sulfate, 0.75 mM; K_i for dichloromethylene diphosphonate, 0.92 mM), whereas the weaker inhibition by ethane-1-hydroxy-1,1-diphosphonate appeared non-competitive.

Neither dichloromethylene diphosphonate nor ethane-1-hydroxy-1,1-diphosphonate had any effect on β-glucuronidase, cathepsin D and esterase.

The effect of a polyphosphate and phosphonates on acid phosphatase

Table IV shows the effect of a polyphosphate of chain length $n = 20$ ($P_{20,i}$) and of various phosphonates on acid *p*-nitrophenyl phosphatase of rat liver lysosomes. Dichloromethylene diphosphonate and undec-10-ene-1-hydroxy-1,1-diphosphonate inhibited when added at 0.1 and 1 mM in the presence of either 0.5 or 2 mM substrate concentration. The greatest effect was seen with 1.0 mM diphosphonate and 0.5 mM substrate. Methylenehydroxydiphosphonate, polyphosphate, condensate I, ethane-1-hydroxy-1,1-diphosphonate and ethane-1-hydroxy-1,1,2-triphosphonate all inhibited slightly but only when added at 1 mM in the presence of 0.5 mM substrate.

The effect of ethane-1-hydroxy-1,1-diphosphonate and dichloromethylene diphosphonate on acid phosphatase of rat calvaria

Dichloromethylene diphosphonate strongly inhibited the acid *p*-nitrophenyl phosphatase activity of an extract of rat calvaria as shown by the Lineweaver-Burk plot in Fig. 4 (K_m for *p*-nitrophenyl phosphate, 0.32 mM, K_i for dichloromethylene diphosphonate, 0.54 mM). Ethane-1-hydroxy-1,1-diphosphonate had no effect.

Discussion

Dichloromethylene diphosphonate inhibited several acid hydrolases more strongly than did ethane-1-hydroxy-1,1-diphosphonate (Fig. 1). In particular

both acid phosphatase and arylsulfatase A were inhibited competitively by dichloromethylene diphosphonate (Figs. 2 and 3). In contrast, ethane-1-hydroxy-1,1-diphosphonate had little or no effect on any of these enzymes. In the case of acid phosphatase, the difference between experiments which showed no effect with ethane-1-hydroxy-1,1-diphosphonate (Fig. 2) compared with those in which slight inhibition occurred (Table IV) may be explained by the use of high ionic strength (0.4 M KCl) in the former.

These results suggest that the ability of dichloromethylene diphosphonate to inhibit bone resorption may be explained in part by a direct effect on lysosomal hydrolases.

Since dichloromethylene diphosphonate causes competitive inhibition of acid phosphatase and arylsulfatase A, any inhibition in vivo will be most pronounced when substrate concentrations are low and dichloromethylene concentrations are high. In order to know whether inhibition is likely to occur in living bone treated with diphosphonates one needs to know what the relative concentrations of dichloromethylene diphosphonate and substrate are likely to be. Apatite crystals in bone of dichloromethylene diphosphonate-treated animals are probably coated with dichloromethylene diphosphonate [16]. During resorption of this bone the diphosphonates may be released to produce relatively high local concentrations of diphosphonate. Bonucci [17] has suggested that crystals released from bone during resorption are phagocytosed and dissolved in the secondary lysosomes of the osteoclast; this could mean that relatively high concentrations of diphosphonates might be present in secondary lysosomes where the final dissolution of crystals occurs. The specific effect of diphosphonates on the skeleton may be related to their binding to crystals.

Of the various phosphonates tested on the acid phosphatase, only dichloromethylene diphosphonate and undec-10-ene-1-hydroxy-1,1-diphosphonate produced strong inhibition, whereas the other compounds had little or no effect. It may be significant that dichloromethylene diphosphonate and undec-10-ene-1-hydroxy-1,1-diphosphonate are the two diphosphonates which most strongly inhibit bone resorption measured by the rise of plasma calcium induced by parathyroid hormone in fasting thyroparathyroidectomized rats (ref. 2, unpublished work). Dichloromethylene diphosphonate is also the most inhibitory of the phosphonates against bone resorption in mouse calvaria in vitro [2]. There is some degree of correlation between the ability of individual phosphonates to inhibit acid hydrolases and bone resorption, suggesting that at least part of their effect in vivo may be explained by a direct effect on acid hydrolases.

Although the majority of these studies were done on enzymes obtained from the light mitochondrial fraction of liver because liver is much easier to fractionate than bone, very similar effects were obtained with dichloromethylene diphosphonate and ethane-1-hydroxy-1,1-diphosphonate on acid phosphatase from rat calvaria (Fig. 4).

An acid pyrophosphatase from potato was not inhibited by either dichloromethylene diphosphonate and ethane-1-hydroxy-1,1-diphosphonate (not shown).

Acknowledgements

This work has been supported by grants from the Swiss National Research Foundation (3.121.73), the U.S. Public Health Service (AM 07266), the Procter and Gamble Co., the National Fund for Research into Crippling Diseases, the Medical Research Council of Great Britain and the Welcome Trust. We are very grateful to Miss M. Leu, B. Haldemann, U. Wirth, Mr. C. Preston and Miss A. Monod for skilled assistance.

References

1 Reynolds, J.J., Minkin, C., Morgan, D.B., Spycher, D. and Fleisch, H. (1972) Calcif. Tissue Res. 10, 302–313
2 Russell, R.G.G., Mühlbauer, R.C., Bisaz, S., Williams, D.A. and Fleisch, H. (1970) Calcif. Tissue Res. 6, 183–196
3 Gasser, A.B., Morgan, D.B., Fleisch, H.A. and Richelle, L.J. (1972) Clin. Sci. 43, 31–45
4 Vaes, G. (1973) Lysosomes in Biology and Pathology (Dingle, J.T. and Fell, H.B., eds.), Vol. 1, pp. 217–253, North-Holland Publishing Co., Amsterdam
5 Vaes, G. (1968) J. Cell Biol. 39, 676–697
6 Vaes, G. (1967) Proceedings of the Third Parathyroid Conference Montreal, Excerpta Medica Int. Congr. Ser. 159, 318–328
7 Morgan, D.B., Monod, A., Russell, R.G.G. and Fleisch, H. (1973) Calcif. Tissue Res. 13, 287–294
8 Schenk, R., Merz, W.A., Mühlbauer, R., Russell, R.G.G. and Fleisch, H. (1973) Calcif. Tissue Res. 11, 196–214
9 Fleisch, H., Russell, R.G.G., Bisaz, S., Mühlbauer, R.C. and Williams, D.A. (1970) Eur. J. Clin. Invest. 1, 12–18
10 de Duve, C., Pressman, B.C., Gianetto, R., Wattiaux, R. and Appelmans, F. (1955) Biochem. J. 60, 604–617
11 Shibko, S. and Tappel, A.L. (1964) Arch. Biochem. Biophys. 106, 259–266
12 Barrett, A.J. (1972) Lysosomes, a Laboratory Handbook (Dingle, J.T., ed.), pp. 110–135, North-Holland Publishing Co., Amsterdam
13 Wöltgens, J. and Ahsmann, W. (1970) Anal. Biochem. 35, 526–529
14 Revel, H.R. and Racker, E. (1960) Biochim. Biophys. Acta 43, 465–476
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Jung, A., Bisaz, S. and Fleisch, H. (1973) Calcif. Tissue Res. 11, 269–280
17 Bonucci, E. (1974) Calcif. Tissue Res. 16, 13–36