the amount of reinnervated muscle fibers. The EZ, however, gains its full original size and shape already after 5 months, i.e. 4 months before reinnervation density is completely restored. Therefore it must be assumed that in a first step the reinnervating motor axon terminals mainly contact the original margin of the EZ. In a second step EPs proliferate exclusively between the borders of the re-established EZ by further ramifying and sprouting of the motor axons. With the methods used it cannot be decided whether the EPs in the transplanted muscle are newly formed or, rather, old ones. However, old EPs can persist for several months in necrotic tissue[1,13]. This fact, together with the finding of the regenerated EZ within its original limits [own observation and previous findings[5,14]] supports the second assumption.

Age (weeks)	3	6	10	16	24	30	36
nEP_T/nEP_C	0.23	0.33	0.71	0.72	0.84	0.88	0.97
lEZ_T/lEZ_C	0.38	0.52	0.74	0.86	1.02	0.95	1.05

Ratios of the mean values of the number (n) of EPs and the length (l) of EZ between the transplanted (nEP_T; lEZ_T) and the contralateral (nEP_C; lEZ_C) muscle. This kind of data presentation eliminates interindividual differences. Preliminary investigations have shown that the EZs of the two superior sternohyoid muscles in the same animal are highly symmetrical in length and position and the number of muscle fibers is virtually identical. This allows the comparison of the number of EPs and the size of the EZ between the transplanted and the contralateral muscle within the same animal.

1 Gutmann, E., and Young, J. Z., J. Anat. *78* (1944) 15.
2 Thompson, N., Plastic reconstr. Surg. *48* (1971) 11.
3 Carlson, B. M., Physiologia bohemoslov. *27* (1978) 387.
4 Hansen-Smith, F. M., Carlson, B. M., and Irwin, K. L., Am. J. Anat. *158* (1980) 65.
5 Carlson, B. M., Otolaryngol. Head Neck Surg. *89* (1981) 192.
6 Allbrook, D., Muscle Nerve *4* (1981) 234.
7 Freilinger, G., Holle, J., and Carlson, B. M. (Eds), Muscle Transplantation, Springer, New York 1981.
8 Mong, F. S. F., and Poland, J. L., IRCS med. Sci. *10* (1982) 81.
9 Ashmore, C. R., Vigneron, P., Marger, L., and Doerr, L., Expl Neurol. *60* (1981) 68.
10 Toop, J., Stain Technol. *51* (1976) 1.
11 Pearse, A. G. E., Histochemistry, 3rd edn, vol. 2, p. 1303. Churchill Livingstone, Edinburgh and London 1972.
12 Müntener, M., Expl Neurol. *77* (1982) 668.
13 Grinnell, A. D., and Herrera, A. A., Progr. Neurobiol. *17* (1981) 203.
14 Bennett, M. R., Florin, T., and Woog, R., J. Physiol., Lond. *238* (1974) 79.

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Effect of halogenmethylenebisphosphonates on bone cells in culture and on bone resorption in vivo

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Summary. Dihalogenmethylenebisphosphonates increase alkaline phosphatase activity and fatty acid oxidation in calvaria cells in culture ($Cl_2MBP > Br_2MBP \cong F_2MBP$). The monohalogen ClMBP and the non-halogenated analogues are less active on phosphatase and inactive on or inhibitory towards fatty acid oxidation. The three dihalogenbisphosphonates and ClMBP inhibit bone resorption in vivo, Cl_2MBP most strongly.
Key words. Bone cells; bisphosphonates; lactate production; alkaline phosphatase; fatty acid oxidation; bone resorption.

Bisphosphonates are analogues of pyrophosphate with a P-C-P bond instead of a P-O-P bond. In vitro they inhibit precipitation and dissolution of calcium phosphate[2-5], and in vivo they slow down bone resorption[3,5-8] and decrease ectopic calcification[2,4] and to some degree bone and cartilage mineralization[6]. While the effect on mineralization appears to be due to a physical chemical action on crystal growth, the mode of action on bone resorption is still unclear. Thus, no correlation has been found between the effect of different bisphosphonates on crystal dissolution in vitro and bone resorption in vivo[9], and because the amounts active in vivo are so small, an action on crystals is unlikely. Consequently, in contrast to mineralization, the action of bisphosphonates on bone resorption is likely to be cellular. Indeed, studies in cell culture have shown that bisphosphonates are taken up by bone cells[10,11] and that they affect various parameters such as cell number[11], production of lactic acid[11], alkaline phosphatase activity[12], fatty acid oxidation[13], collagen[14] and proteoglycan[15] synthesis, PGE_2 production[16] and resorption of bone particles by macrophages[17]. The relevance of these effects of bisphosphonates in cell culture to the inhibitory action of bone resorption is, however, still unclear.

One of the bisphosphonates which has been studied most extensively is Cl_2MBP (for abbreviation of bisphosphonates see table 1). It is a potent inhibitor of bone resorption, but has only a small effect on bone mineralization, both in animals[6] and in humans[18-21]. In vitro Cl_2MBP has the ability to dramatically increase alkaline phosphatase activity of bone cells[12], a property which is shared, although to a lesser degree, by another dihalogen derivative Br_2MBP[9]. Furthermore, Cl_2MBP increases the oxidation of fatty acids[13]. Since these two effects have not been observed with any other of the bisphosphonates investigated up to now, they might be a special characteristic of dihalogenmethylenebisphosphonates. In order to test this hypothesis, the action of Cl_2MBP, Br_2MBP, and F_2MBP as well as that of the monohalogen ClMBP and of nonhalogenated structural analogues have now been investigated.

Since all three dihalogenbisphosphonates have been shown to inhibit bone resorption either in vitro[9,22] or in vivo[9,23], they were compared one with the other, and also with the monohalogen derivative ClMBP, on their effect on bone resorption in vivo. This was carried out in order to assess a possible quantitative relation between their activity on bone resorption and on cell metabolism.

Material and methods. Cell culture. Calvaria from 1-day-old Wistar rats were digested with collagenase. The cells, which included osteoblast-like, periosteal and fibroblast-like cells, but no multinuclear osteoclasts, were then cultured in minimal essential medium containing Earle's salt solution and 10% fetal

Table 1. Bisphosphonates tested

Abbreviations	Full name
Cl_2MBP	Dichloromethylenebisphosphonate
Br_2MBP	Dibromomethylenebisphosphonate
F_2MBP	Difluoromethylenebisphosphonate
ClMBP	Chloromethylenebisphosphonate
MBP	Methylenebisphosphonate
HMBP	Hydroxymethylenebisphosphonate
HEBP	1-Hydroxyethylidene-1,1-bisphosphonate

Table 2. Effect of Cl_2MBP, Br_2MBP and F_2MBP on calvaria cells in culture. The mean ± SEM of n dishes is given in absolute values for the control and as percentage of control

Control	Concentration (μM)	Cell number x 10^{-6}	Lactate production (μmol/10^6cells)	Alkaline phosphatase activity (nmol/min 10^6cells)	Fatty acid oxidation (cpm/10^6cells)
Control	0	0.6337 ± 0.014 (8)	2.68 ± 0.34 (8)	42.3 ± 6.4 (8)	3975 ± 306 (12)
		(%)	(%)	(%)	(%)
Control	0	100.0 ± 1.4 (8)	100.0 ± 2.9 (8)	100.0 ± 5.1 (8)	100.0 ± 3.8 (12)
Cl_2MBP	2.5	96.4 ± 1.7 (7)	90.3 ± 3.1 (7)	125.6 ± 4.4 (8)**	–
	25	86.2 ± 1.5 (8)***	58.7 ± 1.7 (8)***	204.5 ± 10.5 (8)***	110.1 ± 2.5 (10)*
	250	56.6 ± 2.0 (8)***	15.0 ± 2.4 (8)***	545.2 ± 34.9 (8)***	247.5 ± 15.5 (10)**
F_2MBP	2.5	100.5 ± 2.5 (8)	110.1 ± 5.0 (8)	102.2 ± 6.0 (8)	–
	25	99.2 ± 1.3 (8)	87.3 ± 5.9 (8)*	101.8 ± 4.7 (8)	92.0 ± 2.7 (10)
	250	87.6 ± 2.2 (8)***	47.2 ± 2.6 (8)***	236.9 ± 11.0 (8)***	118.5 ± 3.7 (10)**
Br_2MBP	2.5	97.1 ± 1.8 (8)***+	99.0 ± 1.0 (8)+	99.0 ± 5.0 (8)+	–
	25	91.4 ± 1.5 (11)***+	79.6 ± 3.3 (11)***+	144.6 ± 7.7 (8)***+	103.9 ± 2.9 (10)
	250	45.3 ± 2.0 (8)+	75.5 ± 2.0 (8)***+	208.9 ± 9.0 (8)***+	146.9 ± 3.8 (10)**

Values significant by different from control: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$. + These values have been published earlier, see ref. 9.

calf serum, as described elsewhere[11]. Bisphosphonates (abbreviations see table 1), provided by Procter & Gamble Co, Cincinnati, USA, and by Gentili S.p.A. Pisa, Italy, were added during the whole incubation time. At the end of the experiment (day 8) the cell number and the activity of alkaline phosphatase in the cells was determined as described previously[12]. In addition, lactate production and oxidation of fatty acid were measured during the last 16 h of culture (days 7–8). The methods used were those described previously[11,13]. Fatty acid oxidation was measured in a medium with 10% fetal calf serum containing 0.2 μCi/ml [^{14}C(U)]palmitate (spec. act. 800 mCi/mmol), giving a fatty acid concentration of 0.011 mM.

Resorption and mineralization in vivo. Wistar rats of 180 g were treated for 7 days with a dose of 161 μmoles/kg b. wt/day of bisphosphonates, sacrificed on day 8, and the tibia analyzed by microradiography as described earlier[9].

Results and discussion. All three dihalogenbisphosphonates decreased cell number and lactate production and increased the alkaline phosphatase activity and fatty acid oxidation (table 2). The values obtained in this study and those found in an earlier comparison between Cl_2MBP and Br_2MBP[9], indicate that Cl_2MBP is the most potent compound, whereas the activity of Br_2MBP and F_2MBP seemed to be similar.

The monohalogenbisphosphonate ClMBP had a similar effect to Cl_2MBP on cell number and lactate production but was less active on alkaline phosphatase and inactive on fatty acid oxidation (table 3). The analogues containing no halogens, such as MBP, HMBP and HEBP, also differed from the dihalogen-containing compounds in that they induced a smaller or no increase in alkaline phosphatase activity and either no increase or, on the contrary, a decrease of fatty acid oxidation.

High alkaline phosphatase activity is considered as a marker for osteoblast-like cells[24]. The increase of alkaline phosphatase under the influence of dihalogenbisphosphonates might therefore indicate an increase in cells of the osteoblastic type. It has been observed[25] that Cl_2MBP acts mainly on the cells which are released first during collagenase digestion of the calvaria. This cell fraction probably contains preosteoblasts. Inhibition of cell growth did not influence the effect of Cl_2MBP[12], indicating as the mode of action a stimulation of the differentiation towards these cells. In this respect, it might be relevant that cortisol has also been found to increase cellular alkaline phosphatase[26].

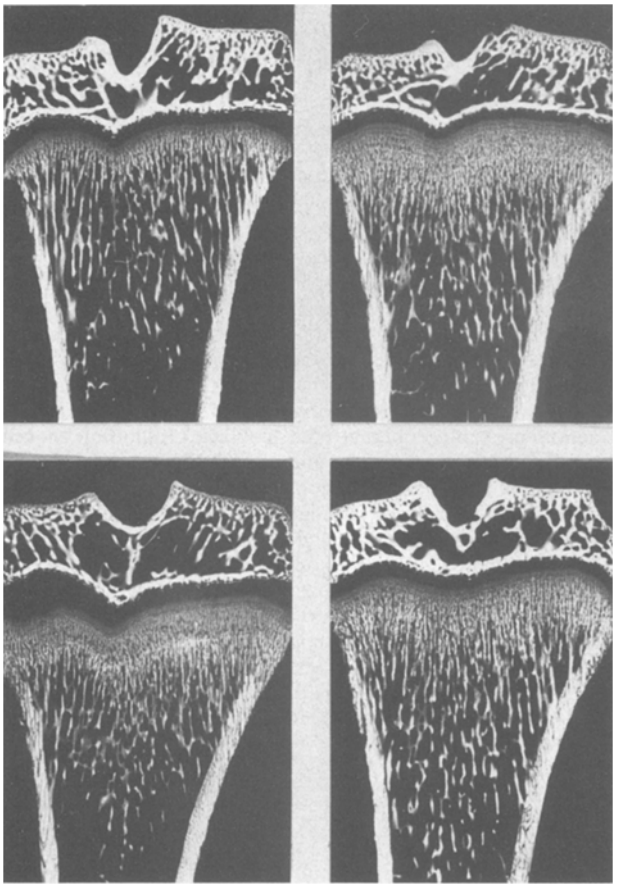
The three dihalogenbisphosphonates inhibited bone resorption in vivo, Cl_2MBP being the most potent (fig.). ClMBP was also active, the effect being comparable with that of Br_2MBP and F_2MBP (not shown). In preliminary findings[23], a small difference seemed to be present between Br_2MBP and F_2MBP which could not be found here. This discrepancy may be due to the relative insensitivity of the technique, so that small changes may be undetectable.

Table 3. Effects of Cl_2MBP, non-halogenated methylenebisphosphonates and HEBP on calvaria cells in culture. The mean ± SEM of n dishes is given in absolute values for the control and as percentage of control

Bisphosphonate	Concentration (μM)	Cell number (x 10^{-6})	Lactate production (μmol/10^6cells)	Alkaline phosphatase activity (nmol/min 10^6cells)	Fatty acid oxidation (cpm/10^6cells)
Control	0	0.4168 ± 0.0054 (16)	3.77 ± 0.14 (16)	58.7 ± 5.1 (24)	3592 ± 268 (20)
		(%)	(%)	(%)	(%)
Control	0	100.0 ± 2.2 (8)	100.0 ± 3.7 (8)	100.0 ± 3.4 (24)	100.0 ± 1.8 (20)
Cl_2MBP	2.5	89.1 ± 2.9 (8)	93.4 ± 3.3 (8)	109.3 ± 12.6 (8)	–
	25	75.3 ± 1.8 (8)***	74.4 ± 2.7 (8)***	184.0 ± 10.9 (16)***	115.1 ± 2.4 (15)***
	250	52.4 ± 1.4 (8)***	40.5 ± 2.4 (8)***	435.0 ± 46.1 (20)***	222.7 ± 10.2 (15)***
ClMBP	2.5	90.6 ± 4.0 (8)*	97.8 ± 2.7 (8)	103.5 ± 10.4 (8)	–
	25	93.2 ± 3.0 (8)*	86.1 ± 3.1 (8)*	104.1 ± 9.0 (8)	92.6 ± 3.2 (11)
	250	73.9 ± 1.4 (8)***	47.9 ± 4.1 (8)***	131.4 ± 6.5 (8)***	106.1 ± 4.3 (20)
MBP	2.5	98.2 ± 3.5 (8)	93.5 ± 3.6 (8)	113.6 ± 4.5 (8)	–
	25	98.0 ± 2.2 (8)	83.9 ± 4.5 (8)*	115.2 ± 9.7 (8)	85.9 ± 2.3 (11)***
	250	109.9 ± 5.1 (8)	58.6 ± 7.0 (8)***	161.5 ± 10.4 (15)***	59.8 ± 6.9 (10)***
HMBP	2.5	96.3 ± 3.2 (8)	79.4 ± 1.7 (8)***	91.5 ± 4.9 (8)	–
	25	93.5 ± 1.5 (8)	36.9 ± 3.4 (8)***	113.8 ± 4.7 (8)	97.6 ± 6.4 (11)
	250	66.4 ± 4.7 (8)***	19.0 ± 2.5 (8)***	92.4 ± 3.7 (8)	114.5 ± 8.4 (10)
HEBP	25	101.0 ± 1.1 (4)	74.3 ± 2.1 (4)***	115.5 ± 4.3 (12)*	86.4 ± 2.8 (11)***
	250	90.5 ± 1.7 (4)*	57.4 ± 2.6 (4)***	156.7 ± 6.4 (12)***	91.7 ± 3.1 (11)*

Values are significantly different from control: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$.

In conclusion, the dihalogenmethylenbisphosphonates Cl_2MBP, and to some extent Br_2MBP and F_2MBP differ from the monohalogen ClMBP and other analogous bisphosphonates without halogens by their stronger potency for increasing the alkaline phosphatase activity and the fatty acid oxidation of calvaria cells in culture. The three dihalogenbisphosphonates show similar activity in inhibiting bone resorption. However, ClMBP and other compounds such as MBP or HMBP, although they are also inhibitors of bone resorption[5,27], show no such cellular effect. There is therefore no general relation between the two processes as regards the effects of bisphosphonates. Thus, the parameters measured may play no role in the effect of bisphosphonates on resorption, or these compounds may act through more than one mechanism, making such a comparison only relatively meaningful.



Contact microradiographs of the proximal tibia in rats. Top left: Control, treated with NaCl; top right: Cl_2MBP; left bottom: Br_2MBP; right bottom: F_2MBP. All bisphosphonates were given for 7 days at a s.c. dose of 161 μmol/kg b. wt/day.

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2 Francis, M.D., Russell, R.G.G., and Fleisch, H., Science *165* (1969) 1264.
3 Fleisch, H., Russell, R.G.G., and Francis, M.D., Science *165* (1969) 1262.
4 Fleisch, H., Russell, R.G.G., Bisaz, S., Mühlbauer R.C., and Williams, D.A., Eur. J. clin. Invest. *1* (1970) 12.
5 Russell, R.G.G., Mühlbauer, R.C., Bisaz, S., Williams, D.A., and Fleisch, H., Calcif. Tissue Res. *6* (1970) 183.
6 Schenk, R., Merz, W.A., Mühlbauer, R.C., Russell, R.G.G., and Fleisch, H., Calcif. Tissue Res. *11* (1973) 196.
7 Reynolds, J.J., Minkin, C., Morgan, D.B., Spycher, D., and Fleisch, H., Calcif. Tissue Res. *10* (1972) 302.
8 Reitsma, P.H., Bijvoet, O.L.M., Verlinden-Ooms, H., and van der Wee-Pals, L.J.A., Calcif. Tissue int. *32* (1980) 145.
9 Shinoda, H., Adamek, G., Felix, R., Fleisch, H., Schenk, R., and Hagan, P., Calcif. Tissue int. *35* (1983) 87.
10 Felix, R., Guenther, H.L., and Fleisch, H., Calcif. Tissue int. *36* (1984) 108.
11 Fast, D.K., Felix, R., Dowse, C., Neuman, W.F., and Fleisch, H., Biochem. J. *172* (1978) 97.
12 Felix, R., and Fleisch, H., Biochem. J. *183* (1979) 73.
13 Felix, R., and Fleisch, H., Biochem. J. *196* (1981) 237.
14 Guenther, H.L., Guenther, H.E., and Fleisch, H., Biochem. J. *196* (1981) 293.
15 Guenther, H.L., Guenther, H.E., and Fleisch, H., Biochem. J. *184* (1979) 203.
16 Felix, R., Bettex, J.-D., and Fleisch, H., Calcif. Tissue int. *33* (1981) 549.
17 Reitsma, P.H., Teitelbaum, S.L., Bijvoet, O.L.M., and Kahn, A., J. clin. Invest. *70* (1982) 927.
18 Delmas, P.D., Chapuy, M.C., Vignon, E., Charhon, S., Briancon, D., Alexandre, C., Edouard, C., and Meunier, P.J., J. clin. Endocr. Metab. *54* (1982) 837.
19 Chapuy, M.C., Charhon, S.A., and Meunier, P.J., Metab. Bone Dis. relat. Res. *4* (1983) 325.
20 Minaire, P., Bérard, E., Meunier, P.J., Edouard, C., Goedert, G., and Pilonchéry, G., J. clin. Invest. *68* (1981) 1086.
21 Delmas, P.D., Charhon, S., Chapuy, M.C., Vignon, E., Briancon, D., Edouard, C., and Meunier, P.J., Metab. Bone Dis. relat. Res. *4* (1982) 163.
22 Rowe, D.J., and Hays, S.J., Metab. Bone Dis. relat. Res. *5* (1983) 13.
23 Felix, R., Fleisch, H., Bonjour, J.-P., and Schenk, R., Calcif. Tissue int. *35* suppl. (1983) 86.
24 Luben, R.A., Wong, G.L., and Cohn, D.V., Endocrinology *99* (1976) 526.
25 Guenther, H.L., Gallagher, J.A., and Fleisch, H., Calcif. Tissue int. *36* (1984) 568.
26 Majeska, R.J., Nair, B.C., and Rodan, G.A., Endocrinology *116* (1985) 170.
27 Trechsel, U., Schenk, R., Bonjour, J.-P., Russell, R.G.G., and Fleisch, H., Am. J. Physiol. *232* (1977) E298–E305.

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Studies on the modulation of the desensitization of the pituitary gland by luteinizing hormone-releasing hormone in the ovariectomized rat

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Summary. In ovariectomized rats the desensitization of the LH cells in vivo, which develops during constant rate infusion of LHRH, 1) does not depend on a concomitant depletion of the pituitary LH stores, 2) proceeds normally when the hypothalamo-pituitary connection has been severed and 3) is a process in which LH itself is not involved.
Key words. Luteinizing hormone release; luteinizing hormone-releasing hormone; ovariectomized rat; desensitization; pituitary stalk section; pituitary autotransplantation; human chorionic gonadotropin.