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Effect of Ethane-1-Hydroxy-1,1-Diphosphonate (EHDP) and Dichloromethylene Diphosphonate (Cl_2MDP) on the Calcification and Resorption of Cartilage and Bone in the Tibial Epiphysis and Metaphysis of Rats

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Young male rats (70–90 g) were treated for various periods with several doses of disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) or disodium dichloromethylene diphosphonate (Cl_2MDP). Effects of treatment on the changes in the thickness, growth and mineralization of proximal growth plate and metaphysis of the tibia were assessed histologically and by microradiography. High doses (10 or 30 mg P/kg/day) of EHDP impaired mineralization of the growth cartilage, which became increased in thickness, and of the osteoid in the metaphysis and diaphysis. Matrix formation continued, although at a diminished rate. High doses (10 or 30 mg P/kg/day) of Cl_2MDP produced a different effect. There was no inhibition of mineralization, but there was a marked impairment of normal metaphyseal remodelling, with persistence of columns of calcified cartilage. Resorption at the periosteal surface in the metaphysis was also inhibited, so that the metaphysis became club-shaped. Osteoclasts were present in large numbers in the metaphysis, but their appearance was abnormal and similar to that seen in human osteopetrosis.

Key words: Diphosphonates — Cartilages — Bones — Calcification — Resorption.

Des jeunes rats mâles (70–90 g) ont été traités pendant des laps de temps variables avec différentes doses d'éthane-1-hydroxy-1,1-diphosphonate disodique (EHDP) ou de dichlorométhylène diphosphonate disodique (Cl_2MDP). Les effects de ces traitements sur l'épaisseur, la croissance et la minéralisation du cartilage de conjugaison et de la métaphyse du tibia ont été étudiés par des méthodes histologiques et microradiographiques. Des doses élevées (10 ou 30 mg P/kg/jour) d'EHDP empêchent la minéralisation de l'ostéoide dans la métaphyse et la diaphyse et celle du cartilage épiphysaire dont l'épaisseur augmente. La formation de la matrice osseuse se poursuit, mais à une vitesse réduite. Le Cl_2MDP à doses élevées (10 ou 30 mg P/kg/jour) produit un effet différent. On n'observe aucune inhibition de la minéralisation, mais une diminution nette du remodàlement normal de la métaphyse, où des colonnes de cartilage calcifié persistent. Il y a également, pour la métaphyse, une inhibition de la résorption à la surface périostale provoquant une métaphyse en forme de massue. Dans la métaphyse on note la présence de nombreux ostéoclastes d'apparence anormale qui ressemblent à ceux observés dans l'ostéopétrose humaine.

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Junge männliche Ratten (70–90 g) wurden während verschiedenen Zeitabschnitten mit unterschiedlichen Dosen von Dinatrium Äthan-1-hydroxy-1,1-Diphosphonat (EHDP) oder Dinatrium Dichloromethylen-Diphosphonat (Cl_2MDP) behandelt. Die Wirkung der Behandlung auf die Veränderungen in Dicke, Wachstum und Mineralisation der proximalen Wachstumsplatte und Metaphysis der Tibia wurde histologisch und mikroradiographisch untersucht. Hohe Dosen (10 oder 30 mg P/kg/Tag) von EHDP beeinträchtigten die Mineralisation des Wachstumsknorpels, welcher breiter wurde, und des Osteoids in der Metaphyse und Diaphyse. Die Matrixbildung ging weiter, jedoch weniger schnell. Hohe Dosen (10 oder 30 mg P/kg/Tag) von Cl_2MDP hatten eine andere Wirkung. Die Mineralisation wurde nicht gehemmt, aber der normale Metaphysenumbau wurde merklich gestört, wobei Säulen von verkalktem Knorpel bestehen blieben. Die Resorption auf der Periostoberfläche in der Metaphyse wurde ebenfalls gehemmt, so daß die Metaphyse keulenförmig wurde. Osteoclasten traten in großen Mengen in der Metaphyse auf, aber ihr Aussehen war abnormal und glich demjenigen, das bei der menschlichen Osteopetrose beobachtet wird.

Introduction

In the previous paper (Russell *et al.*, 1973) we described changes produced by the two diphosphonates, ethane-1-hydroxy-1,1-diphosphonate disodium salt [$CH_3(OH)C(PO_3HNa)_2$, EHDP] and dichloromethylene diphosphonate disodium salt [$Cl_2C(PO_3HNa)_2$, Cl_2MDP] on the chemical composition of rat femora and on quantitative histological measurements made in the tibial diaphysis. In this paper we report the changes observed in the epiphysis and the metaphysis.

Methods

Male Wistar rats weighing 70–90 g were used. The rats were bred in this laboratory (Department of Pathophysiology, Berne) and from the time of weaning were fed a diet containing 1.28% Ca and 0.85% P (diet 194, Nafag AG, Gossau, Switzerland).

The first two experiments were carried out on animals treated with 0, 0.01 and 0.1 mg P/kg/ of Cl_2MDP or EHDP given daily subcutaneously for 10 days and with doses of 0, 1, 10 and 30 mg P/kg/day. These animals were from experiments 2 and 3 as described in the previous paper (Russell *et al.* 1973). Further experiments were performed in which rats were treated for various periods up to 14 days with doses of 1, 10 and 30 mg P/kg/day of EHDP or Cl_2MDP. EHDP and Cl_2MDP were obtained from the Procter and Gamble Company, Cincinnati, USA. The treatments were allocated at random to the animals within each experiment. The diphosphonates were made up in water (except for the lower doses of 1 mg P/kg/day or less, which were made up in 0.9% NaCl) and the pH was adjusted to 7.4 with HCl or NaOH, as appropriate. The daily dose was given in a volume of 0.2 ml per 100 g body weight, except for the 30 mg P/kg dose, which was given as 2×0.3 ml per 100 g body weight. During the experiment, all the animals had free access to food and distilled water. One and two days before the beginning of the treatment period, each animal received 15 mg of oxytetracycline/kg body weight intraperitoneally daily.

Histological Technique

At the end of the experiment the rats were killed and the tibia removed from the fibula. The entire tibia was fixed and dehydrated, without prior decalcification, in increasing concentrations of ethanol. The bone was block-stained at the same time by including 0.5% basic fuchsin in the ethanol solutions (Schenk, 1965). The whole tibia was then embedded in methylmethacrylate, with addition of Plastoid N (Burkhardt, 1966). Frontal or sagittal sections of the head of the tibia were cut with a precision saw and ground to a thickness of 70–80 μ. These sections were used for the preparation of microradiographs and for measurements made by fluorescence microscopy. In addition, thin undecalcified sections (5 μ) were cut on a special microtome. These sections were stained by the trichrome method of Goldner (1938) and for calcium phosphate by the method of Krutsay (1963) and Schenk *et al.* (1969). Measurements of longitudinal growth and thickness of the epiphyseal plates were performed under the micro-

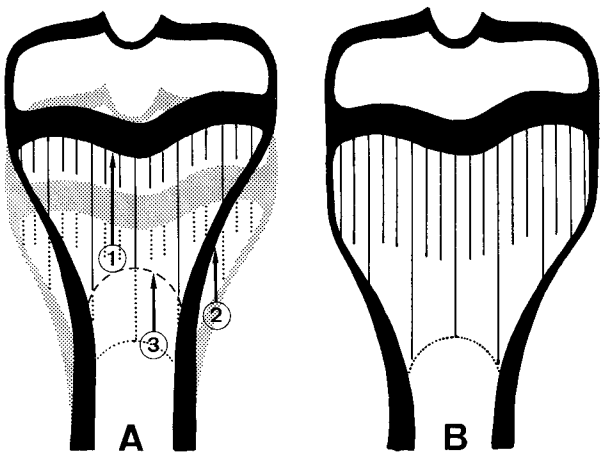

Fig. 1A and B. Schematic representation of the remodelling that takes place during growth of the proximal end of the tibia. In A), the outlines of a section in a frontal plane are indicated by the dotted area. The situation after a growth period of 14 days is superimposed in black. Resorption of calcified tissue occurs at three different sites: 1. Resorption of longitudinal (intercolumnar) calcified cartilage septa by multinucleated chondroclasts, so that only about one in three of the original septa remain. 2. Osteoclastic resorption along the periosteal surface of the metaphysis. 3. Enlargement of the marrow cavity by osteoclastic resorption of metaphyseal cancellous bone. B) represents a diagram of the configuration of a metaphysis which would be expected to result from a complete inhibition of osteoclastic and chondroclastic resorption. Note the close resemblance to the microradiograph of a section from an animal treated with Cl_2MDP (see Fig. 2D)

scope at a magnification of 40. Longitudinal growth rate was calculated by measuring the distance between the epiphyseal margin (i.e. upper surface) of the growth cartilage and the uppermost tetracycline-labelled trabeculae in the metaphysis. By taking the upper rather than the lower margin of the epiphyseal cartilage plate as a fixed point for the measurement of growth in length, errors due to the increasing width of the growth cartilage (Table 1) under treatment with the high doses of EHDP are avoided. The microradiographs were used to locate the calcification front in the epiphyseal cartilage and to estimate the mineral density in the trabecular bone of the metaphysis.

Assessment of Changes in Metaphyseal Remodelling

To understand the changes observed in the metaphysis and shown in Fig. 1, it is helpful to consider what happens during normal bone growth in the metaphysis.

Growth in length of long bones is based on two different processes: a) the formation of new bone trabecula in the primary cancellous bone of the metaphysis by the epiphyseal plate; b) the remodelling of the cone-shaped metaphysis by a process which involves a complex pattern of bone formation and bone resorption along the periosteal and endosteal surfaces, respectively, of the cortical bone.

For these processes to take place correctly, resorption of calcified tissue has to occur at various sites. Firstly, there is continual *resorption of calcified cartilage* along the metaphyseal side of the growth plate. This process is performed by multinucleated giant cells which resemble osteoclasts, but which are better referred to as chondroclasts. This resorption reduces to about one third of their original number the number of calcified cartilaginous septa separating the columns of hypertrophic cartilage cells in the zone of provisional calcification. The remaining calcified cartilage septa serve as a scaffold for the deposition of fibrous bone and therefore persist as central cores within the primary trabecula. It is interesting to note that, unlike the

longitudinal cartilage septa, the transverse cartilage walls, which separate the chondrocytes in individual cell columns, remain unmineralised and are ultimately removed during invasion of metaphyseal capillaries and accompanying cells (see Fig. 7 A). The resorption of calcified cartilage which reduces the number of mineralised longitudinal cartilage septa, is achieved by multinucleated giant cells, whereas the vascular invasion on the metaphyseal side of the growth plate relies on chondrocytes, perivascular cells and eventually endothelial cells (Schenk *et al.*, 1967).

Secondly, remodelling of the cone-shaped metaphysis of the proximal end of the tibia involves *bone resorption* by osteoclasts along the periosteal surface and bone formation on the endosteal surface. Near the epiphyseal plate, the periosteal surface of the metaphysis is formed by the tips of bone trabecula (see Figs. 2, 5 and 6). Further down towards the diaphysis, a layer of true cortical bone is present. In both regions, considerable numbers of multinucleated osteoclasts are present and their activity results in retention of the conical shape of the metaphysis during longitudinal growth.

Finally, resorption takes place as the marrow cavity of the diaphysis enlarges in length. This is achieved by osteoclasts located around the lower tips of the trabecula. Some osteoclastic resorption is also involved in the remodelling of the central area of cartilage and bone in the metaphysis.

Longitudinal growth in the tibial metaphysis proceeds with a considerable speed in young animals such as used in this study. The growth rate can be calculated from the values given in Table 1 and amounts to approximately 300 μ/day. Based on this value, the outlines of the metaphysis before and after 14 days of growth may be superimposed (Fig. 1 A) to illustrate the large amount of formation and destruction of cartilage and bone necessary for the complex process of growth of length. Clearly, such a system offers an excellent opportunity for a histological analysis of the effects of compounds which are thought to influence the formation and resorption of calcified tissues. For instance, if resorption and remodelling processes are completely blocked, a cylindrical deformation of the metaphyseal region and an increased density in the cancellous bone can be predicted, as illustrated schematically in Fig. 1 B. Similarly, any effects on the production of matrix in these tissues and on its subsequent mineralisation should also be easily detectable. These potentialities are well illustrated in our studies with EHDP and Cl_2MDP.

Results

Effects of Cl_2MDP

Measurements of Longitudinal Growth Rate and of the Thickness of the Epiphyseal Plate. In Table 1, the distance between the top of the epiphyseal plate and the uppermost trabecular bone labelled with tetracycline amounted to an average of 3870 μm in NaCl-treated control animals. From this value, the mean thickness of the epiphyseal plate (505 μm) in the control animals killed at the beginning of the experiment has to be subtracted in order to assess the actual length deposited in the 11 day interval. It is assumed that the epiphyseal plate does not significantly change in thickness in the untreated normal animals during the experiment. From these measurements, the longitudinal growth rate in the proximal metaphysis in the tibia of normal rats amounted to an average of 306 μm/day. In the animals treated with Cl_2MDP, growth remained normal up to and including a dose of 10 mg P/kg, but an inhibition of growth occurred in animals treated with 30 mg P/kg/day. This dose also provoked a significant increase in the thickness of the epiphyseal plate which, however, was still small compared with the animals treated with the same dose of EHDP.

Inhibition of Bone and Cartilage Resorption by Cl_2MDP. In the microradiographs, an increase in density of the metaphyseal trabecular bone became evident after injections of 1 mg or more daily for 14 days. Doses of 0.01 and 0.1 mg P/kg/day for

Table 1. Effect of Cl_2MDP and EHDP on the thickness of the epiphyseal plate and on the growth in length of the proximal metaphysis of the tibia. The animals were injected with tetracycline on each of two successive days and then with diphosphonates for the following ten days. They were killed the day after the last injection. Experiments 2 and 3 denote experiments described in the accompanying paper (Russel *et al.*, 1973)

Exp.	Treatment	No. of animals	Thickness of epiphyseal plate (μ i.e. mm^{-3}) mean ± S.E.	Distance between top of epiphyseal plate and uppermost tetracycline-labelled trabecular bone (μ i.e. mm^{-3}) mean ± S.E.
2	Control (NaCl only)	15	505 ± 16	3857 ± 76
	Cl_2MDP (mg P/kg/day)			
	1	6	500 ± 20	3636 ± 81
	10	6	500 ± 20	3439 ± 127**
	30	6	727 ± 74***	2454 ± 163***
	EHDP (mg P/kg/day)			
	1	5	581 ± 36	3981 ± 213
	10	6	1848 ± 128***	2863 ± 160***
	30	6	1515 ± 69***	1939 ± 56***
3	Control (NaCl only)	4	507 ± 34	3912 ± 88
	Cl_2MDP (mg P/kg/day)			
	0.01	6	544 ± 33	3848 ± 157
	0.1	6	560 ± 31	3606 ± 153
	EHDP (mg P/kg/day)			
	0.01	6	560 ± 23	3949 ± 103
	0.1	6	515 ± 30	3909 ± 152
2+3	Controls combined from 2 and 3	19	505 ± 14	3870 ± 62

*, ** and *** denote difference from control (NaCl alone) significant at less than 5%, 1% and 1‰ level, respectively.

10 days produced no visible effects. Fig. 2 shows microradiographs of the tibiae from control rats treated with 1, 10 or 30 mg Cl_2MDP as P/kg/day subcutaneously for 14 days. At doses of 10 mg P/kg/day the newly-formed part of the metaphysis took on a cylindrical shape and at the highest dose, 30 mg P/kg/day, there was a further increase in bone density and a more pronounced cylindrical shape. With the high doses (10 and 30 mg) it is remarkable how closely the microradiographic appearances (Fig. 2C, D) resemble those predicted (see Fig. 1B), if cartilage and bone resorption in the metaphysis were completely inhibited.

Even in the low power microradiograph, it can be seen that the gain in bone density in the metaphysis is due mainly to an increase in the number rather than the thickness of individual trabecula. This is more clearly seen in thin microtome sections, such as Fig. 3, which shows the zones of cartilage calcification and of vascular invasion and primary ossification. In control animals (Fig, 3A), the pattern

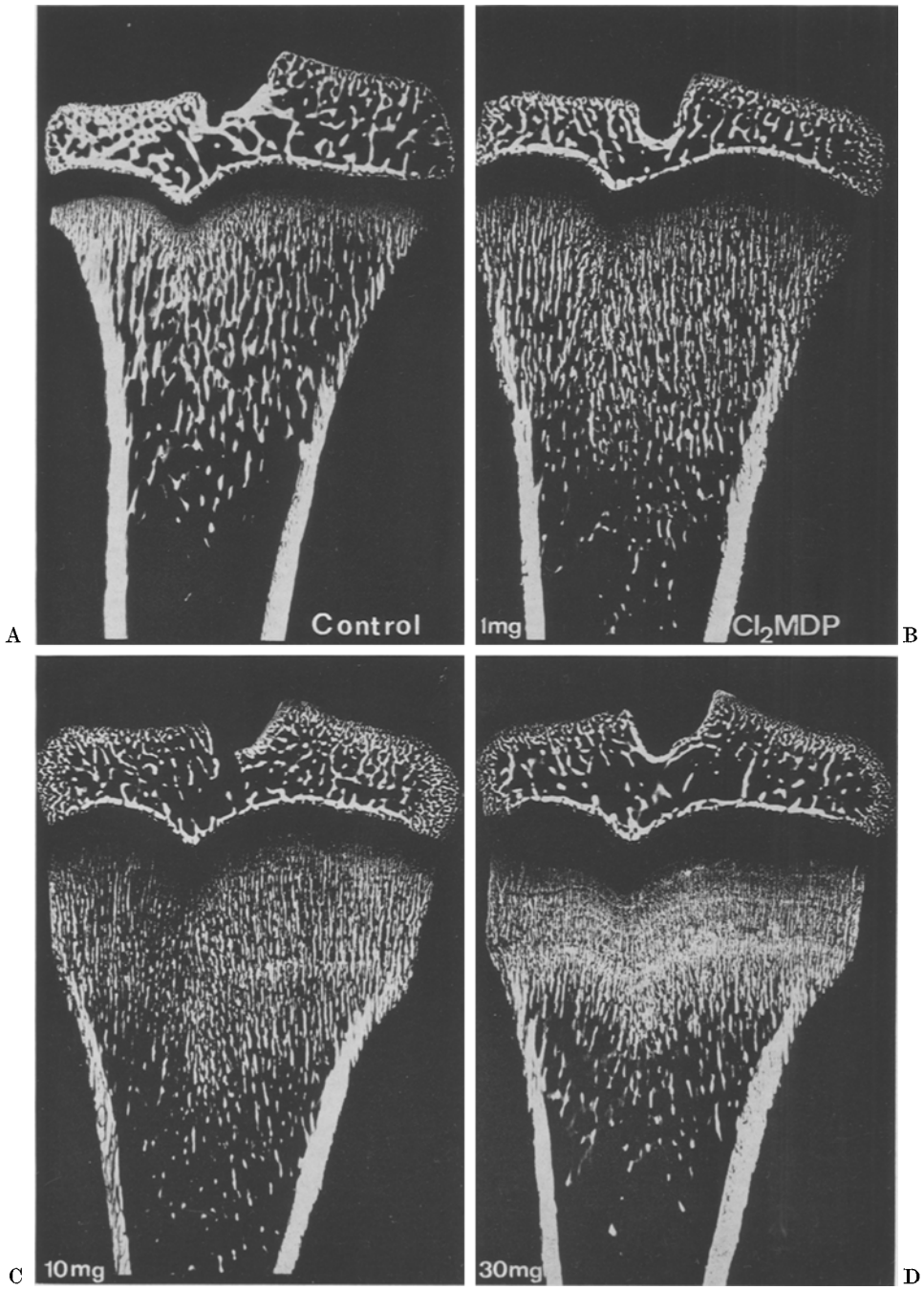

Fig. 2 A—D. Microradiographs of tibiae from control rats (A) and rats treated with Cl_2MDP at 1 (B), 10 (C) and 30 mg P/kg/day (D) subcutaneously for 14 days

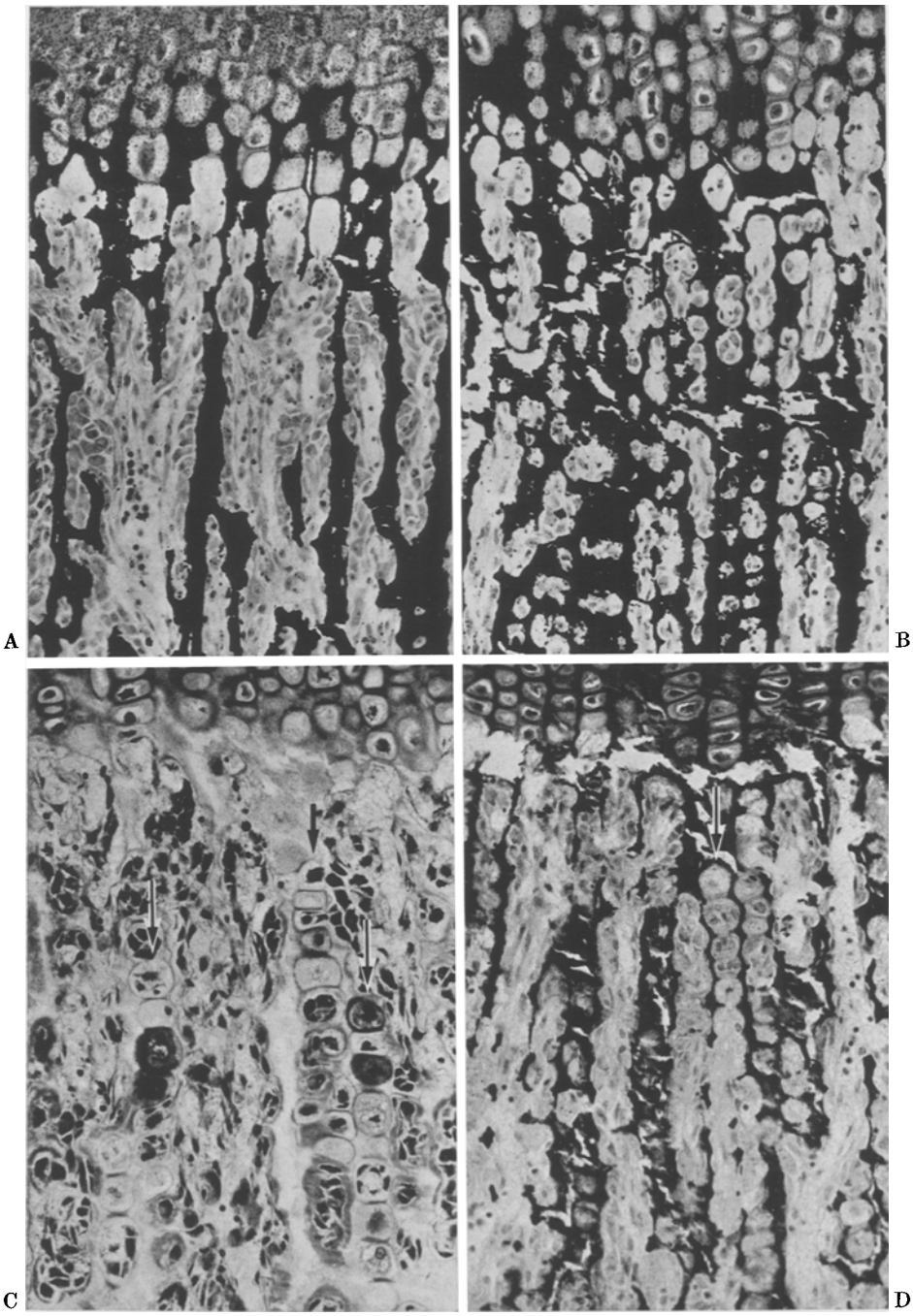

Fig. 3 A—D. Undecalcified microtome sections (5 μ) through the zones of cartilage mineralisation, vascular invasion and ossification of the growth plate. A Control, von Kossa reaction, counterstained with methylgreen-pyronine. B Cl_2MDP 30 mg P/kg/day s. c. for 10 days, von Kossa-methylgreen-pyronine. C Cl_2MDP 30 mg P/kg/day s. c. for 10 days, Goldner stain. D) Cl_2MDP 30 mg P/kg/day s. c. for 10 days, PAS-alcianblue stain. In C and D, arrows indicate the position of persisting columns of chondrocytes which are completely surrounded by calcified cartilage, $\times$ 150

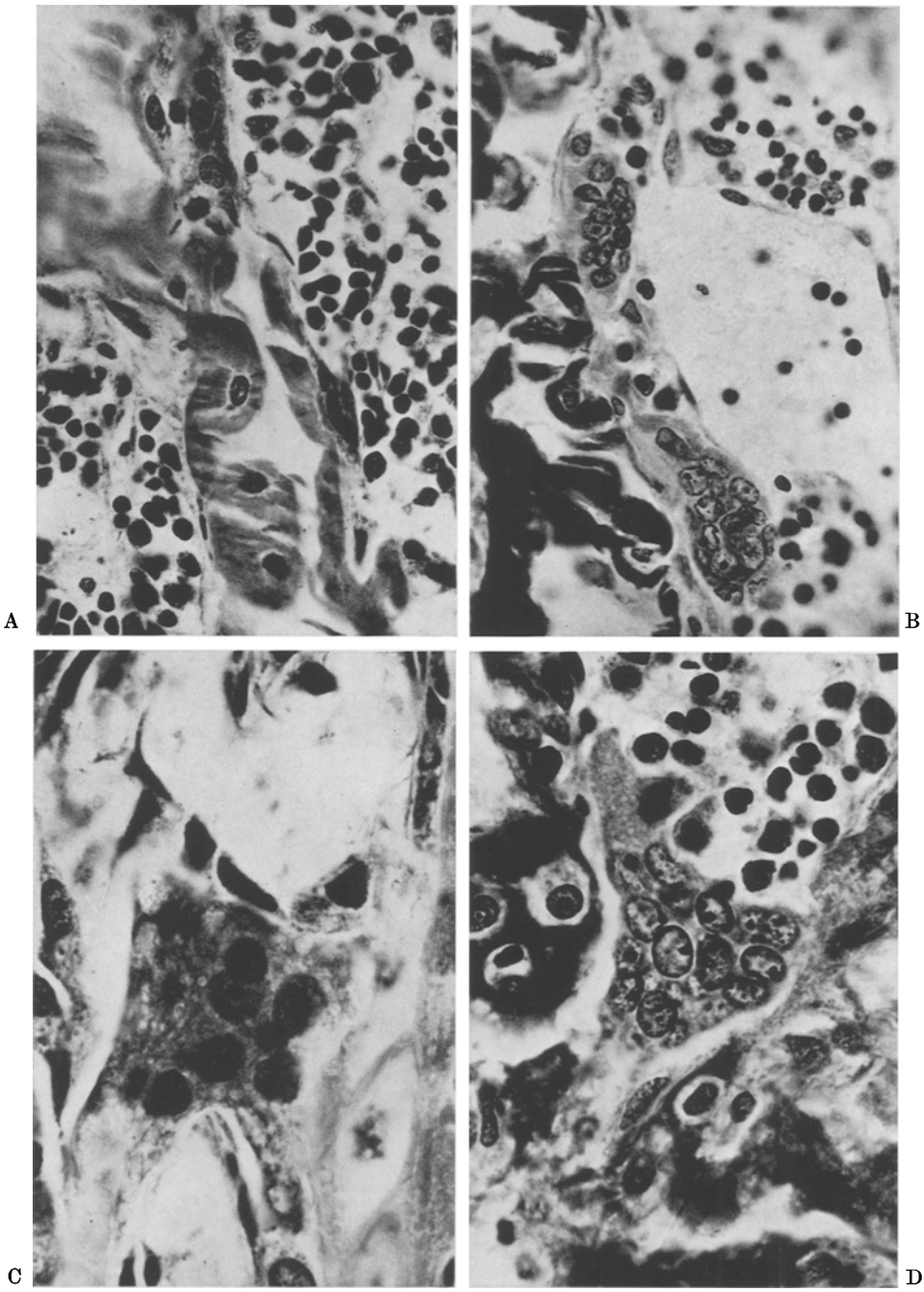

Fig. 4 A—D. Osteoclasts in the metaphysis of control animals (A and C) and after treatment with Cl_2MDP 10 mg P/kg/day s. c. for 10 days (B and D). Goldner stain. A and B, ×450; C and D, ×750

of cartilage mineralisation is characteristically concentrated on the longitudinal intercolumnar septa. The cell columns are being invaded by capillaries, and the numbers of mineralised cartilage septa are being reduced in the metaphysis by the action of chondroclasts. The calcified cartilage septa which remain become lined by osteoblasts and subsequently coated by woven bone.

In Fig. 3B, striking differences are seen in an animal treated for 10 days with Cl_2MDP at 30 mg P/kg/day subcutaneously. Firstly, there is an increase in amount of calcified tissue. This is due to the persistence of almost all the calcified intercolumnar septa. The narrow interspaces between these septa maintain the shape and size of the former lacunae of the chondrocytes. Most of the chondrocytes as well as the surrounding unmineralised cartilaginous matrix have disappeared. Some of the intercolumnar spaces are invaded by capillaries, others not.

In the sections stained according to Goldner (1938) (Fig. 3C) or with PAS-alcian blue (Fig. 3D), one sees occasional columns of chondrocytes completely surrounded by calcified cartilage persisting within the metaphysis. These columns are surrounded by capillaries and are partially enclosed in newly-formed bone trabecula. Such columns of cells are not seen in normal rats.

Changes in Osteoclasts (Figs. 3 and 4). In spite of the clear inhibition of bone and cartilage resorption, there was no reduction in the number of osteoclasts in the metaphysis of animals treated with Cl_2MDP. On the contrary, counts of the approximate numbers of multinucleated osteoclasts in the ten and fourteen days experiments suggest numbers higher than usual. Osteoclasts were consistently found along the periosteal surface of the metaphysis both in control animals and after treatment with Cl_2MDP at doses up to 10 and 30 mg P/kg. The cytological features of the osteoclasts, however, were different under Cl_2MDP (Fig. 4). There was a marked increase in the number of nuclei per cell and a lack of the cytoplasmic vacuoles normally found in actively resorbing cells. Moreover, the cells were less readily stained by acid dyes (Fig. 4, B and D) and the cell margins were more regular than cells in normal bone. Occasionally, large cells consisting merely of a "pocketful of nuclei" could be found (Fig. 4D).

Effects of EHDP

Measurements of Longitudinal Growth Rate and of the Thickness of the Epiphyseal Plate. There were no detectable differences in the thickness of the epiphyseal plate and the longitudinal growth rate between animals treated with doses of EHDP up to 1 mg P/kg/day and the control animals (Table 1). With higher doses (10–30 mg P/kg/day) of EHDP, however, all animals showed severe disturbances of growth and mineralisation. The epiphyseal plate was much thicker than in the controls, and the longitudinal growth rate was reduced. The inhibition of growth was more marked with the higher dose, but not the enlargement of the epiphyseal plate.

Effect on Mineralisation in the Epiphysis and Metaphysis. Microradiographs confirmed that EHDP at 10 and 30 mg/kg/day caused a widening of the unmineralised part of the growth cartilage (Fig. 5C and D). The micrographs also revealed a series of transverse lines of low mineral density in the trabecular bone of the metaphysis (Figs. 5C and 6B, C and D). These lines were spaced at intervals of about 300 μ, which is equivalent to the daily growth in length. The number of lines seen equalled the number of days for which EHDP had been injected at 10 mg P/day,

at least up to about 5 days (Fig. 6B, C and D). Later the transverse bands give way to an appearance compatible with a complete block of mineralisation.

Histological examination confirms the presence of such regularly spaced alternating bands of high and low density (Fig. 8B). In the low density areas, small spicules of calcified cartilage remain, whereas in the zones of higher density, the number and thickness of the trabecula is increased by the persistence of more calcified cartilage septa partially covered by mineralised bone. After 5 days of EHDP at 10 mg P/kg/ daily, there is no longer any calcified cartilage present in the growth plate (Fig. 8C).

The inhibition of mineralisation did not occur uniformly over the whole extent of the zone of calcification. This led to a variable, steplike delineation of the arrest of mineralisation, particularly well seen in microradiographs after 5 and 10 days of treatment (Figs. 5C and 6D). It seems that each band of low mineral density corresponds to inhibition of mineralisation in the first few hours after each injection of EHDP and is followed by a recovery period.

After EHDP at 30 mg/kg, given as one daily injection, the number of transverse bands seen are reduced to about one, suggesting that mineralisation is completely inhibited after the first day of treatment.

Early Effects on Mineralisation. The early events in the inhibition of mineralisation by EHDP were studied in rats given two injections of 15 mg EHDP/kg spaced 12 h apart and killed 12 h after the second injection (Fig. 7). The normal appearance in von Kossa-stained undecalcified microtome sections from control animals is shown in Fig. 7A. The mineral is concentrated in the longitudinal intercolumnar septa and extends upward into the growth cartilage to a height of about 3–4 chondrocytes. As the diameter of the lacuna surrounding a hypertrophic chondrocyte approximates to about 35 μ, the daily advance in the front of calcification and subsequent vascular erosion will correspond to a column of about 8–10 hypertrophic cells (i.e. 300 μ). In the animals treated with 2×15 mg EHDP for 24 h, there was a complete absence of calcification between the columns of chondrocytes in the growth cartilage (Fig. 7B). The penetration of capillaries into epiphyseal plate has halted at the level of the intercolumnar cartilage septa which had undergone mineralisation immediately before treatment started. The zone of hypertrophic cartilage cells retained its columnar arrangement, except for a small layer of chondrocytes next to the tips of the calcified bars. These cells had disintegrated and their lacunae had collapsed, presumably because there is no longer any mechanical reinforcement of the intercolumnar septa by newly deposited mineral.

In the metaphysis, numerous osteoblasts lined the surfaces of the calcified cartilage septa present before treatment with EHDP started. Under normal conditions, these cells produce layers of woven (fibrous) bone, which mineralises within a few hours after the organic matrix has been laid down. Therefore, only very thin osteoid seams, barely detectable by light microscopy, are present in the control animals. Since the von Kossa reaction stains both calcified cartilage and bone equally, the bone formed in this region is evident only as a thickening of the primary trabecula (Fig. 7C). After two doses of 15 mg P of EHDP in 24 h, mineralisation of this newly-formed bone matrix was completely blocked. The cores of calcified cartilage in the center of the primary trabecula were covered by unmineralised osteoid seams much wider than in untreated animals (Fig. 7D). Thus the two cardinal features

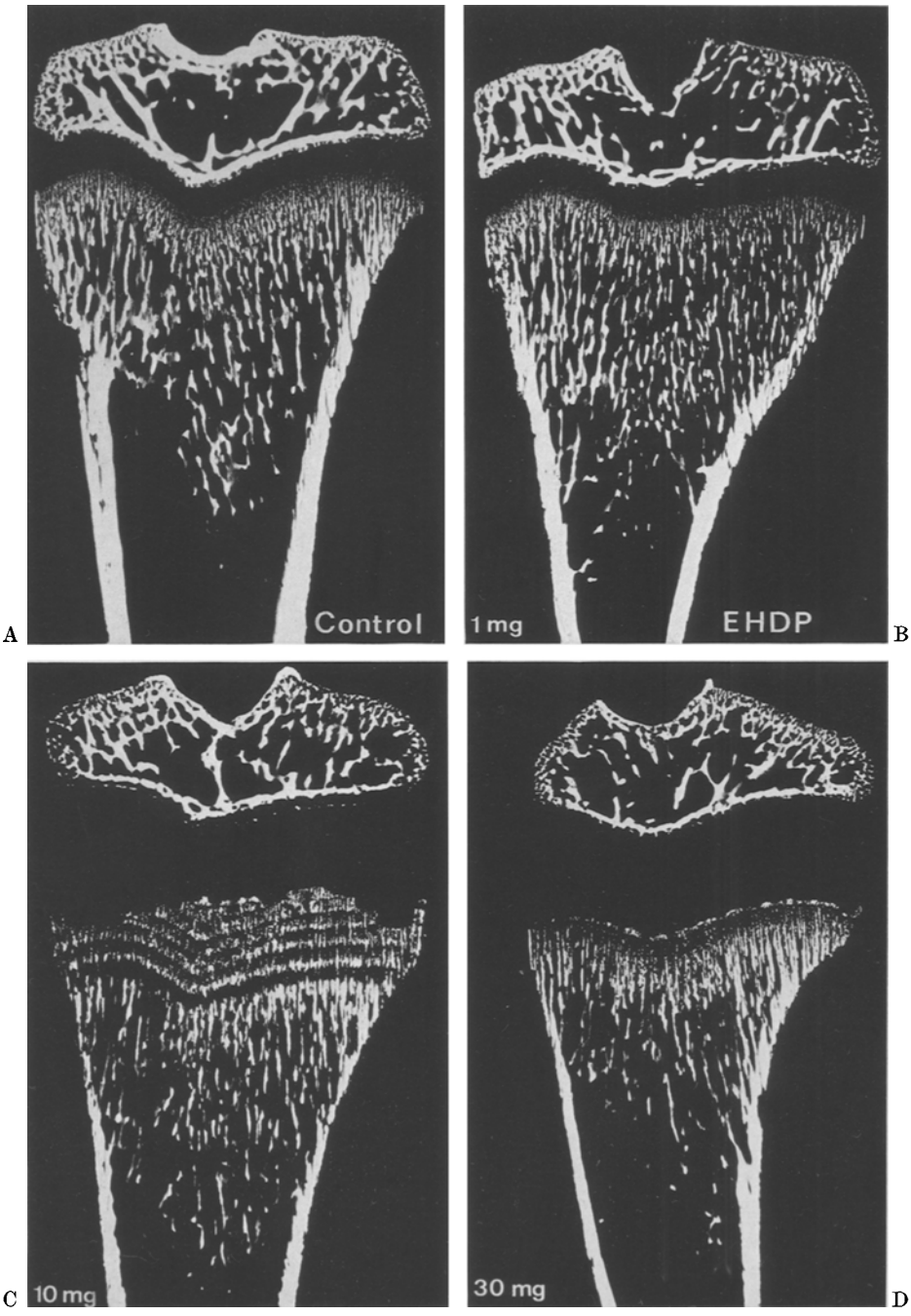

Fig. 5 A—D. Microradiographs of tibiae from control rat (A) and rats treated with EHDP at 1 (B) 10 (C) and 30 mg P/kg/day s. c. (D) for 10 days, ×7,5

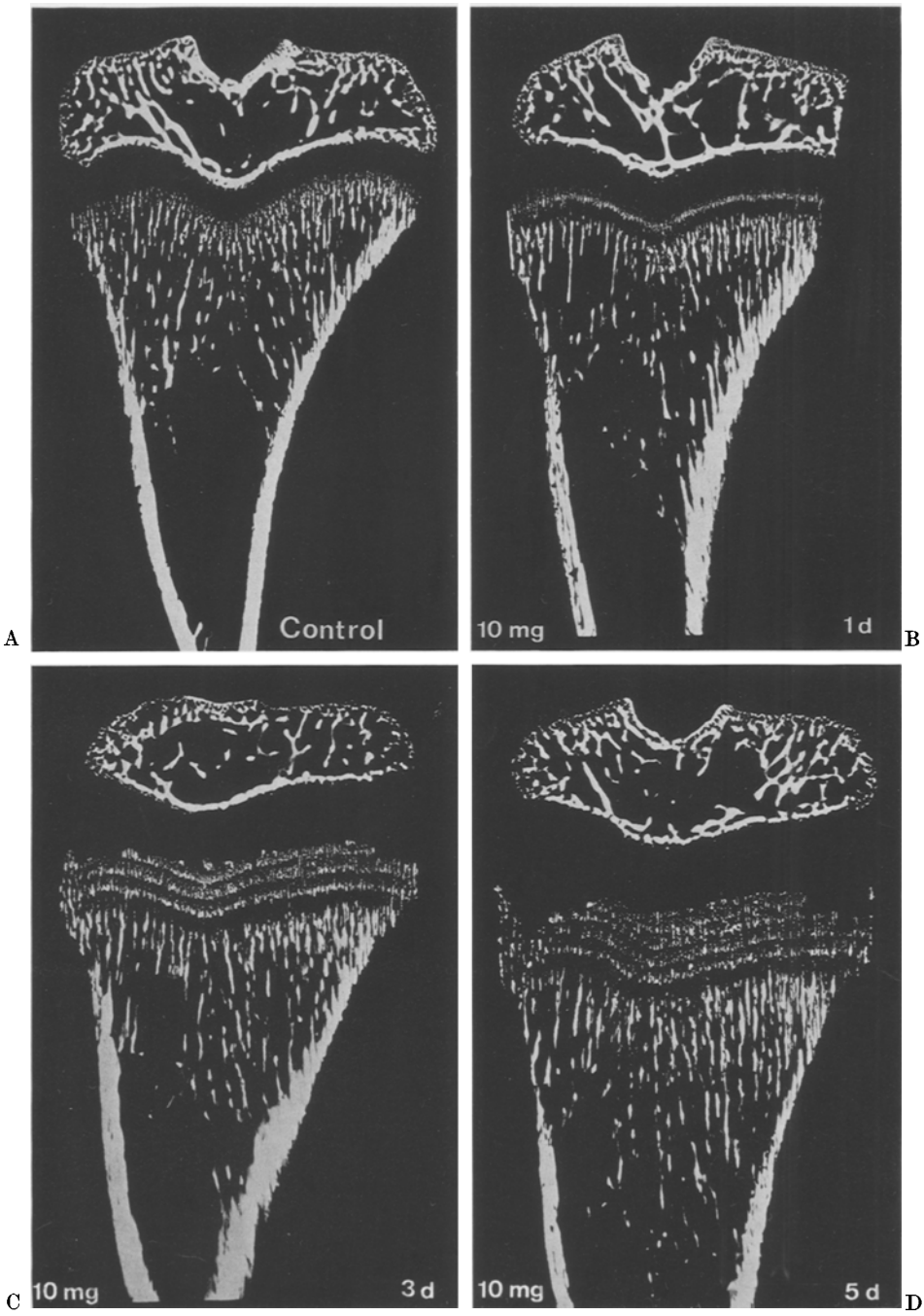

Fig. 6A—D. Microradiographs of tibiae from control rat (A) and rats treated with EHDP at 10 mg P/kg/day s.c. for 1 (B), 3 (C) and 5 days (D), ×7,5

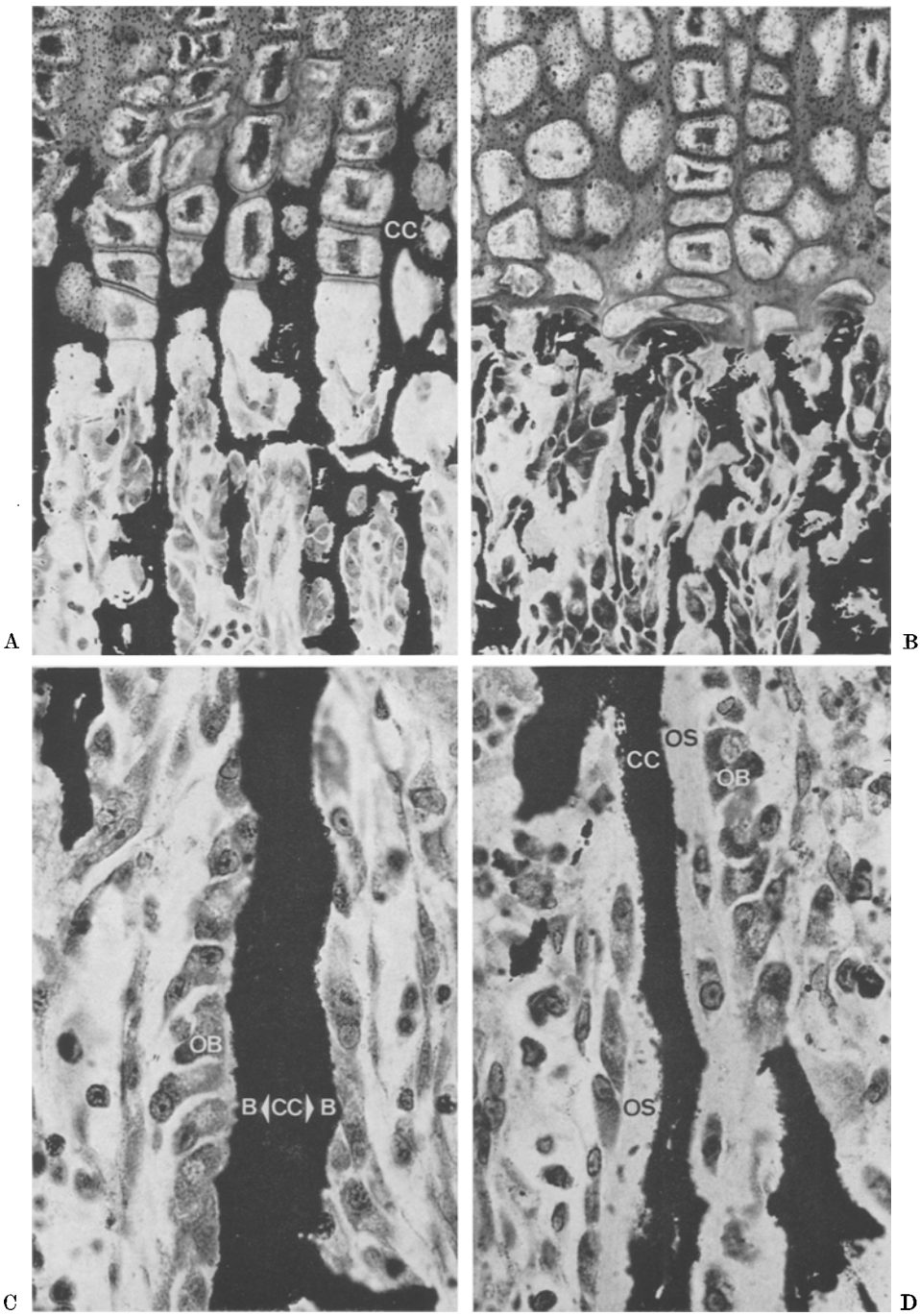

Fig. 7 A—D. Undecalcified microtome sections (5 μ) through the metaphyseal part of the growth apparatus of a control animal (A and C) and an animal (B and D) 24 h after two s. c. injections (given at 0 and 12 h) of 15 mg P of EHDP/kg. *B* mineralised bone, *CC* calcified cartilage, *OB* osteoblast, *OS* osteoid seam. Calcified cartilage and bone are not distinguishable by this staining method, the approximate borderline between these tissues is indicated by triangular marks in C). Von Kossa-methylgreen-pyronine stain. A) and B), ×225; C) and D), ×600

of impaired calcification, i.e. lack of mineralisation in the hypertrophic cartilage zone and formation of wide osteoid seams in the metaphysis, are both present within 24 h of giving 2×15 mg P of EHDP.

Renewed Vascular Invasion of Growth Cartilage. After 5 days treatment with EHDP at 10 and 30 mg P/kg/day the border line between the cartilage and the metaphyseal trabecula was no longer regular. This was because the vascular invasion of the growth cartilage had only been temporarily interrupted and a renewed vascular invasion of the growth plate began again after the 5th day in most animals. In the absence of mineral, the vascular ingrowth was irregularly distributed along the metaphyseal aspect of the growth plate (Figs. 8C and D, 9A and C) and led to tongue-shaped invasion areas in which large capillary knots and accompanying connective tissue were present (Fig. 9C). The zones of erosion were separated by cartilage septa containing several cell columns (Figs. 8D, 9A–C). The borderline of cartilage which was subjected to this kind of erosion underwent characteristic histological changes. In sections stained with pyronine (Fig. 9C), there was first a loss of metachromatic material in the ground substance accompanied by cellular disintegration and collapse of the walls of the empty lacunae. In the vicinity of the blood vessels entering the areas of renewed cartilage removal, a homogeneous intercellular substance was found which was lined by osteoblasts and included cells resembling osteocytes. This material closely resembled osteoid and did not contain any traces of calcified cartilage or bone mineral. The buds of vascular invasion were completely unrelated to the steplike pattern of mineralisation arrest seen in the corresponding microradiographs (Fig. 9B). The renewed vascular invasion of non-mineralised cartilage resembles that seen in established vitamin D deficiency rickets (Irving, 1965).

Effects on Resorption Seen in Microradiographs. EHDP did not produce the great increase in metaphyseal density seen with Cl_2MDP (Figs. 2, 5 and 6). However, the mineralised bands between the transverse low density lines did show an increase in density and an array of numerous fine longitudinal trabecula similar to inhibition of remodelling of calcified tissues in the growth apparatus of the tibia, those seen in the animals treated with Cl_2MDP (Figs. 5C, 6B, C, D). In addition, the general shape of the metaphysis tended to resemble the cylindrical form noted under treatment with Cl_2MDP. Thus, EHDP also seemed to produce an inhibition of resorption of calcified cartilage and bone, but this was less obvious than with Cl_2MDP and only occurred at doses at which mineralisation disturbances were also evident.

Discussion

EHDP and Cl_2MDP both inhibit the growth and dissolution of hydroxyapatite crystals *in vitro* (Fleisch *et al.*, 1968; 1969; 1970; Francis, 1969; Francis *et al.*, 1969; Russell *et al.*, 1970) and block the calcification induced by large doses of vitamin D in the aorta and kidneys of rats (Fleisch *et al.*, 1970). Both compounds also inhibit bone resorption in tissue culture (Fleisch *et al.*, 1969; Russell *et al.*, 1970) and prevent the bone loss that occurs after immobilisation of one hind limb of rats (Mühlbauer *et al.*, 1971; Michael *et al.*, 1971). In earlier studies some differences were noted between the effects of EHDP and Cl_2MDP. Thus, when tested against bone resorption, Cl_2MDP was more potent than EHDP at equivalent doses (Russell *et al.*, 1970).

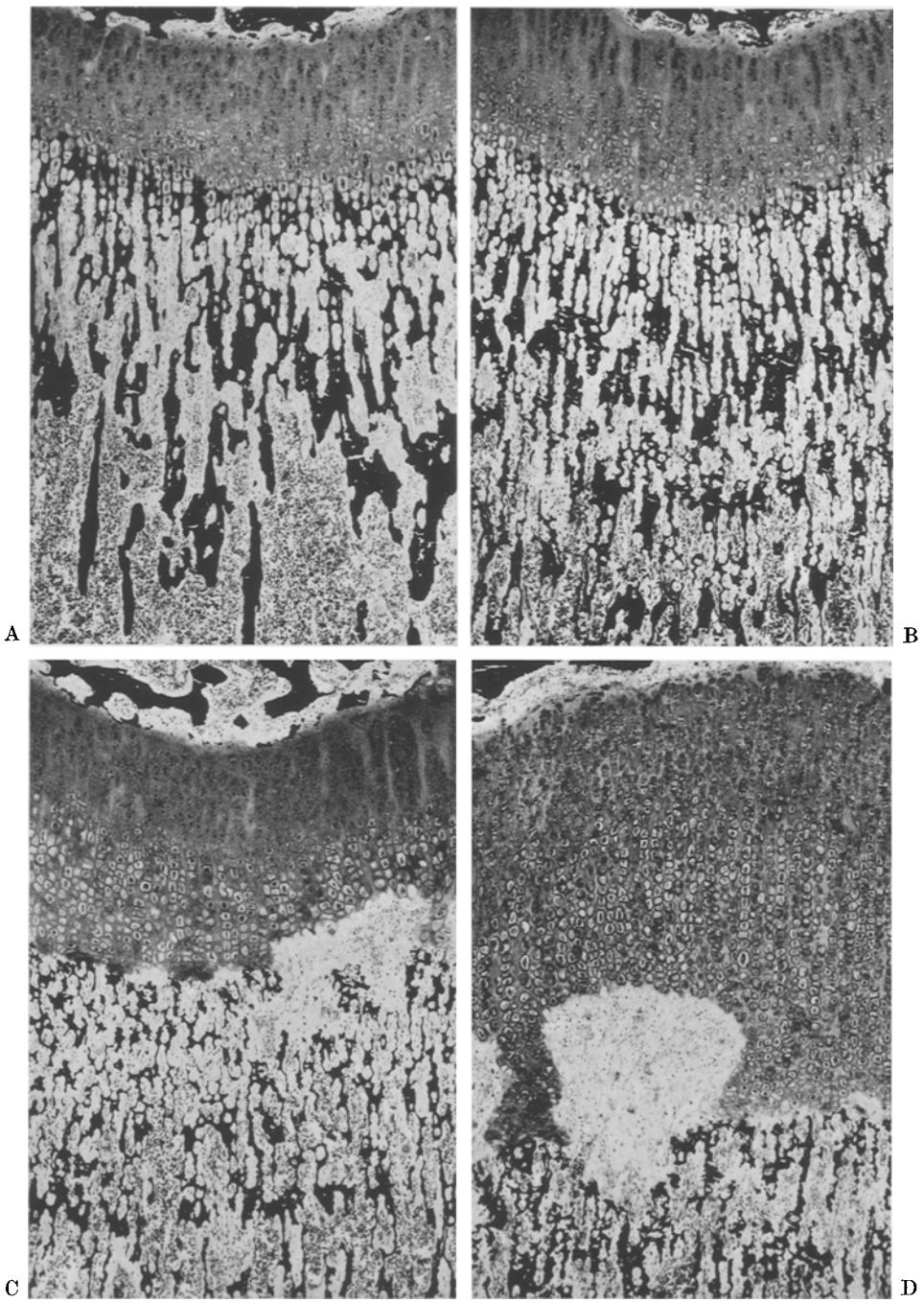

Fig. 8 A—D. Growth plate and metaphysis in control rat (A) and rats treated for 3 (B), 5 (C) and 8 days (D) with 10 mg P of EHDP/kg/day s.c. Von Kossa-methylgreen-pyronine stain, ×45

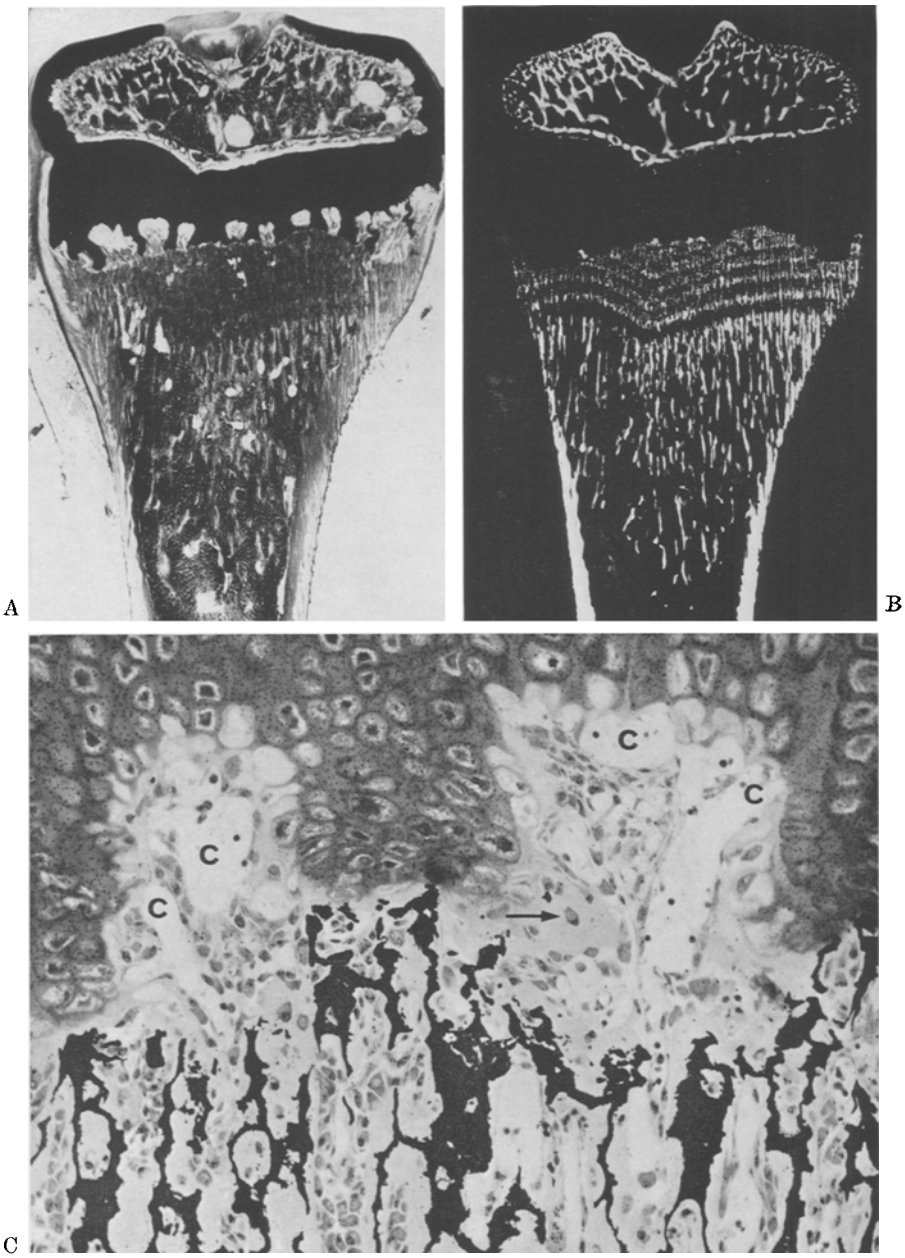

Fig. 9 A—C. Renewed vascular invasion of growth cartilage after 10 days treatment with 10 mg P of EHDP/kg/day s.c. A) Plastic embedded ground section, stained with basic fuchsin. B) Corresponding microradiograph. C) Undecalcified microtome section, von Kossa-methylgreen-pyronine stain. *C* capillaries, arrow points to an osteocyte completely surrounded by osteoid. A) and B), ×7,5; C), ×225

Also, it soon became clear that at high doses EHDP produced mineralisation defects in bone, whereas Cl_2MDP did not. Since both EHDP and Cl_2MDP are under consideration as potential therapeutic agents against human diseases of excessive calcification (Basset *et al.*, 1969; Weiss *et al.*, 1971; Russell *et al.*, 1972) and excessive bone resorption (Smith *et al.*, 1971), it seemed important to define the differences between the compounds in greater detail.

In the present study, some very clear differences between the effects of EHDP and Cl_2MDP have been demonstrated. The higher doses of EHDP consistently produced a marked inhibition of mineralisation in the epiphysis and metaphysis of rat tibiae. In contrast, the most striking effect of Cl_2MDP at high doses was an with very little evidence for direct inhibition of mineralisation processes. EHDP also produced some inhibition of cartilage remodelling but, at the same doses, inhibition of mineralisation was the most obvious effect.

The inhibition of mineralisation with high doses of EHDP seemed to occur very soon after injection of EHDP. With a dose of 15 mg P/kg/day given at 12 h intervals the inhibition of mineralisation occurred immediately and was sustained. However, with the lower dose of 10 mg P/kg/day, the inhibition of mineralisation was not maintained throughout the 24 h between injections. Each injection seemed to produce a band of poorly mineralised cartilage and bone in the metaphysis. After 3–5 days of injections there were therefore 3–5 bands in the metaphysis, spaced at intervals of about 300 μ, equivalent to the daily growth in length calculated from tetracycline measurements. This suggests that shortly after one subcutaneous injection of EHDP at this dose, there is an increased concentration of EHDP present in the intercellular fluid and that this leads to a temporary inhibition of mineralisation. Mineralisation resumes as the concentration of EHDP falls again before the next injection.

With Cl_2MDP the largest effects were seen on resorption of cartilage and bone. The increase in thickness of the growth cartilage after 30 mg P/kg was not accompanied by any disturbance of cartilage mineralisation in the hypertrophic zone or in the osteoid of the metaphysis. This suggests that the widening of the epiphyseal plate is probably not due to the same mechanism as EHDP. It is possible that the persistence of densely-packed mineralised cartilage septa during treatment with Cl_2MDP prevents the normal processes of vascular invasion of the calcified cartilage. This might alter the resorption of the epiphyseal cartilage and result in its increased thickness. The club-shaped metaphyses produced by large doses of Cl_2MDP are reminiscent of the metaphyses seen in human osteopetrosis, in which bone remodelling is also impaired. It is also interesting to note that the changes in osteoclasts under Cl_2MDP resemble those seen in human osteopetrosis in that they contain more nuclei than usual and contain fewer intracellular vesicles (R. Schenk, unpublished observations). In both osteopetrosis and Cl_2MDP-treated animals, there is a failure of normal bone resorption, and the changes in osteoclasts in both situations may represent a response to their inability to digest mineralised bone.

The marked differences between the effects of EHDP and Cl_2MDP on mineralisation and resorption are difficult to explain in terms of present knowledge. Both diphosphonates inhibit the crystal growth and dissolution of apatite crystals, and it is tempting to ascribe their effects on bone to these phenomena. However, the slightly greater effect of EHDP compared with Cl_2MDP on crystal growth (Fleisch

et al., 1970) seems insufficient to account for the marked difference between the two on mineralisation *in vivo*. Furthermore, EHDP is stronger then Cl_2MDP as an inhibitor of crystal dissolution *in vitro*, whereas, *in vivo*, Cl_2MDP is stronger than EHDP against bone resorption in tissue culture (Russell *et al.*, 1970; Reynolds *et al.*, in press) and in living animals (Mühlbauer *et al.*, 1971; Russell *et al.*, 1970). It is possible that the distribution of EHDP and Cl_2MDP within bone is not the same and that this accounts for the discrepancy between the effect of these two diphosphonates *in vitro* and *in vivo*. However, both diphosphonates might work by other mechanisms, e.g. by effects on cell metabolism, on membrane transport, or on enzymes (Fleisch *et al.*, 1972). Further work is needed to define the mode of action of EHDP and Cl_2MDP on bone.

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References

Bassett, C. A. L., Donath, A., Macagno, F., Preisig, R., Fleisch, H., Francis, M. D.: Diphosphonates in the treatment of myositis ossificans. Lancet **1969 II**, 845,

Burkhardt, R.: Präparative Voraussetzungen einer klinischen Histologie des menschlichen Knochenmarkes. Blut **14**, 30—46 (1966).

Fleisch, H., Bonjour, J.-P., Morgan, D. P., Reynolds, J. J., Schenk, R., Smith, R., Russel, R. G. G.: Diphosphonates. In: Endocrinology, 1971. 430–443. London: Heinemann Medical Publishers (1972).

Fleisch, H., Russell, R. G. G., Bisaz, S., Casey, P. A., Mühlbauer, R. C.: The influence of pyrophosphate analogues (diphosphonates) on the precipitation and dissolution of calcium phosphate *in vitro* and in *vivo*. Calc. Tiss. Res. **2**, Suppl. 10–10A (1968).

Fleisch, H., Russell, R. G. G., Bisaz, S., Mühlbauer, R. C., Williams, D. A.: The inhibitory effect of phosphonates on the formation of calcium phosphate crystals *in vitro* and on aortic and kidney calcification *in vivo*. Europ. J. clin. Invest. **1**, 12–18 (1970).

Fleisch, H., Russell, R. G. G., Francis, M. D.: Diphosphonates inhibit hydroxyapatite dissolution *in vitro* and bone resorption in tissue culture and *in vivo*. Science **165**, 1262–1264 (1969).

Francis, M. D.: The inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates. Calc. Tiss. Res. **3**, 151–162 (1969).

Francis, M. D., Russell, R. G. G., Fleisch, H.: Diphosphonates inhibit formation of calcium phosphate crystals *in vitro* and pathological calcification *in vivo*. Science **165**, 1264–1266 (1969).

Goldner, J. A.: A modification of the Masson trichrome technique for routine laboratory purpose. Amer. J. Path. **14**, 237–243 (1938).

Irving, M. H.: The blood supply of the growth cartilage and metaphysis in rachitic rats. J. Path. Bact. **89**, 461–471 (1965).

Krutsay, M.: Methode zur Darstellung einzelner Calciumverbindungen in histologischen Schnitten. Acta histochem. (Jena) **15**, 189–191 (1963).

Michael, W. R., King, W. R., Francis, M. D.: Effectiveness of diphosphonates in preventing osteoporosis of dissue in the rat. Clin. Orthop. **78**, 271–276 (1971).

Mühlbauer, R. C., Russell, R. G. G., Williams, D. A., Fleisch, H.: The effects of diphosphonates on "immobilisation osteoporosis" in rats. Europ. J. clin. Invest. **1**, 336–344 (1971).

Russell, R. G. G., Mühlbauer, R. C., Bisaz, S., Williams, D. A., Fleisch, H.: The influence of pyrophosphate, condensed phosphates, phosphonates and other phosphate compounds on the dissolution of hydroxyapatite *in vitro* and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomised rats. Calc. Tiss. Res. **6**, 183–196 (1970).

Russell, R. G. G., Smith, R., Bishop, M., Price, D., Squire, D.: Treatment of myositis ossificans with a diphosphonate. Lancet **1972 I**, 10–11.

Russell, R. G. G., Kisling, A., Casey, P. A., Fleisch, H., Thornton, J., Schenk, R., Williams, D. A.: Effect of diphosphonates and calcitonin on the chemistry and quantitative histology of rat bone. Calc. Tiss. Res., **11**, 179–195 (1973).

Schenk, R.: Zur histologischen Verarbeitung von unentkalkten Knochen. Acta anat. (Basel) **60**, 3–19 (1965).

Schenk, R., Merz, W. A., Müller, J.: A quantitative histological study on bone resorption in human cancellous bone. Acta anat. (Basel) **74**, 44–53 (1969).

Schenk, R., Spiro, D., Weiner, J.: Cartilage resorption in the tibial epiphyseal plate of growing rats. J. Cell Biol. **34**, 275–291 (1967).

Smith, R., Russell, R. G. G., Bishop, M.: Diphosphonates and Paget's disease of bone. Lancet **1971 I**, 945–947.

Weiss, I. W., Fisher, L., Phang, J. M.: Diphosphonate therapy in a patient with myositis ossificans progressiva. Ann. intern. Med. **74**, 933–936 (1971).