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## Effects of 1,25- and 24,25-dihydroxyvitamin D<sub>3</sub> on bone formation in the cichlid teleost *Sarotherodon mossambicus*

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**Summary.** 1,25-Dihydroxyvitamin D<sub>3</sub> and 24,25-dihydroxyvitamin D<sub>3</sub> have antagonistic effects on the acellular bone of the tilapia *Sarotherodon mossambicus*. 1,25-Dihydroxyvitamin D<sub>3</sub> inhibits the activity of the lining osteoblasts. Prolonged administration leads to demineralization of the bone matrix.

Injection of 24,25-dihydroxyvitamin D<sub>3</sub> induces structural signs of greatly enhanced appositional bone growth within three days. No effects were observed on the mineral content of pre-existing bone.

Both 1,25- and 24,25-dihydroxyvitamin D<sub>3</sub> may have distinct, but different physiological functions in fish.

**Key words:** Bone (acellular, teleosts) – 1,25-dihydroxyvitamin D<sub>3</sub> – 24,25-dihydroxyvitamin D<sub>3</sub> – Osteoblasts

Although fish liver is known as a rich source of vitamin D, the physiological significance in fish of vitamin D and its metabolites is hardly understood. In higher vertebrates, one or possibly more vitamin D<sub>3</sub> metabolites function as endocrine factors regulating calcium and phosphate metabolism, in particular intestinal uptake and bone development and mineralization. In mammals and birds, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) is considered the metabolically most active vitamin D<sub>3</sub> metabolite, at least in respect to the stimulating action of vitamin D<sub>3</sub> on intestinal calcium and phosphate uptake and its calcium-mobilizing action on bone (Barnes and Lawson 1978). It is formed by hydroxylation of vitamin D<sub>3</sub> in the liver at the 25-position, and subsequently at the 1-position in the kidneys (Fraser and Kodicek 1970; Holick et al. 1971).

Another hydroxylation in the kidneys leads to the formation of 24R,25-dihydroxyvitamin D<sub>3</sub> (24,25(OH)<sub>2</sub>D<sub>3</sub>) (Holick et al. 1972). However, the biological significance of this metabolite is still in discussion. For mammals and birds it has

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been reported to be required for normal skeletal development, to increase bone mineralization, and to inhibit PTH secretion (Malluche et al. 1980; Canterbury et al. 1978). However, hydroxylation at the 24-position has also been considered a step in a degradative pathway of vitamin D metabolites (Holick et al. 1976).

Data on the action of vitamin D<sub>3</sub> and its metabolites in fish are scarce, and evidence that these compounds are physiologically important in fish is circumstantial. In fish blood, transport proteins for 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> have been identified (Hay and Watson 1976, 1977). Furthermore, the presence of 25(OH)D<sub>3</sub>-1-hydroxylase has been demonstrated in kidneys of various fish species (Henry and Norman 1975; Kenny et al. 1977), indicating that 25(OH)D<sub>3</sub> can be converted into 1,25(OH)<sub>2</sub>D<sub>3</sub>. Effects of administration of vitamin D<sub>3</sub> or its metabolites reported so far show some similarity with those reported for mammals and birds. Vitamin D<sub>3</sub> induces hypercalcemia in catfish (Ahmad and Swarup 1979; Swarup and Srivastav 1982). 1,25(OH)<sub>2</sub>D<sub>3</sub> increases plasma phosphate in eels (MacIntyre et al. 1976), and has a calcium-mobilizing action on bone of immature eels (Lopez et al. 1977). This metabolite was further shown to stimulate intestinal calcium uptake in eels (Chartier et al. 1979) and the tilapia *Sarotherodon mossambicus* (Flik et al. 1982). For 24,25(OH)<sub>2</sub>D<sub>3</sub> it has been reported that it did not affect plasma phosphate in eels (MacIntyre et al. 1976).

To further explore the possible biological significance of vitamin D<sub>3</sub> metabolites in lower vertebrates, we compared the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> on osteoblastic bone formation in the teleost *Sarotherodon mossambicus*.

## Materials and methods

*Experimental animals.* Two groups of sexually mature male *Sarotherodon mossambicus* from laboratory stock were used. Body weights varied from 20 to 30 g. They were kept in 100 l tanks with circulating tap water at 26°C and were fed daily with tropical fish food (Tetramin) and minced beef heart.

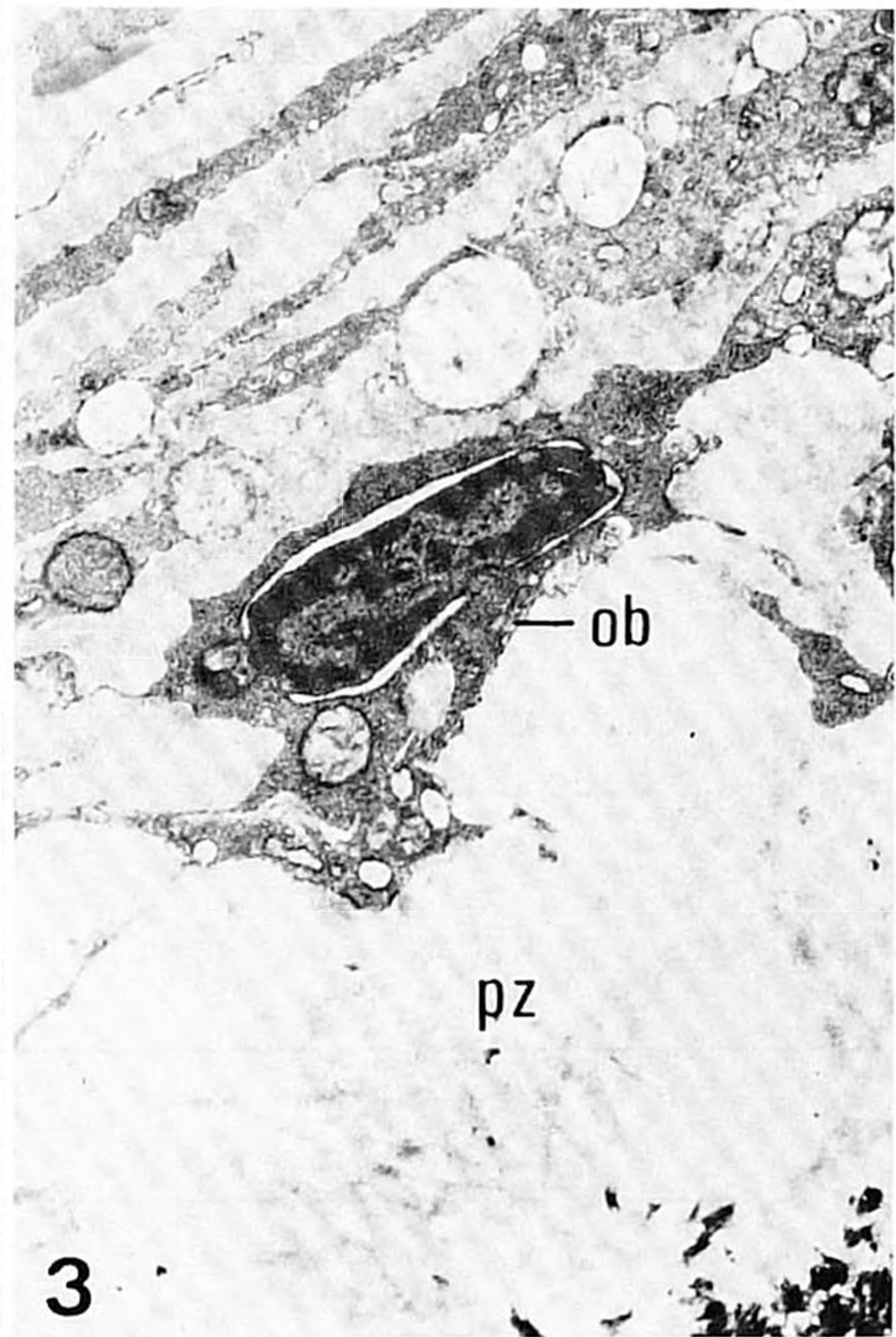
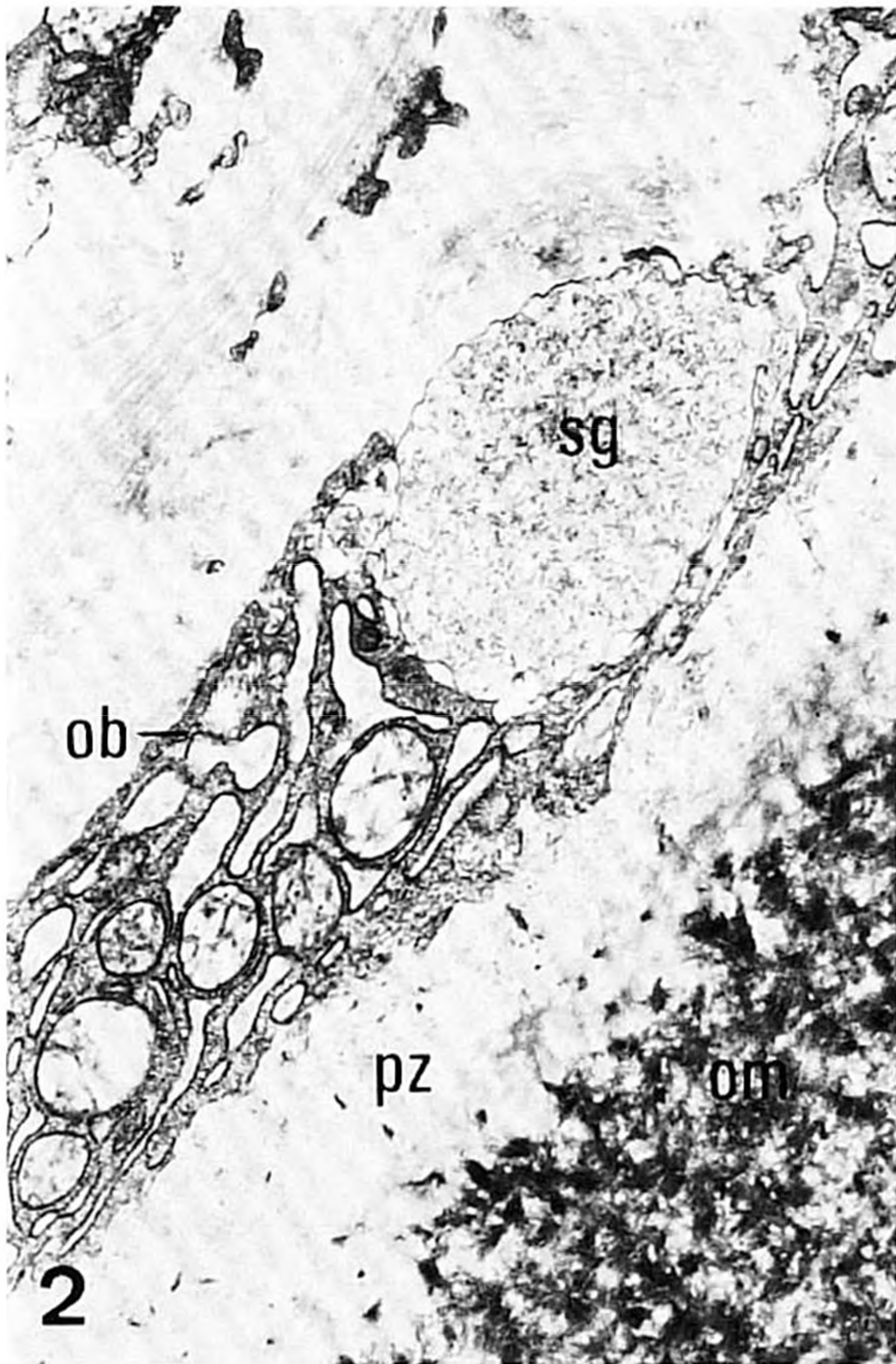
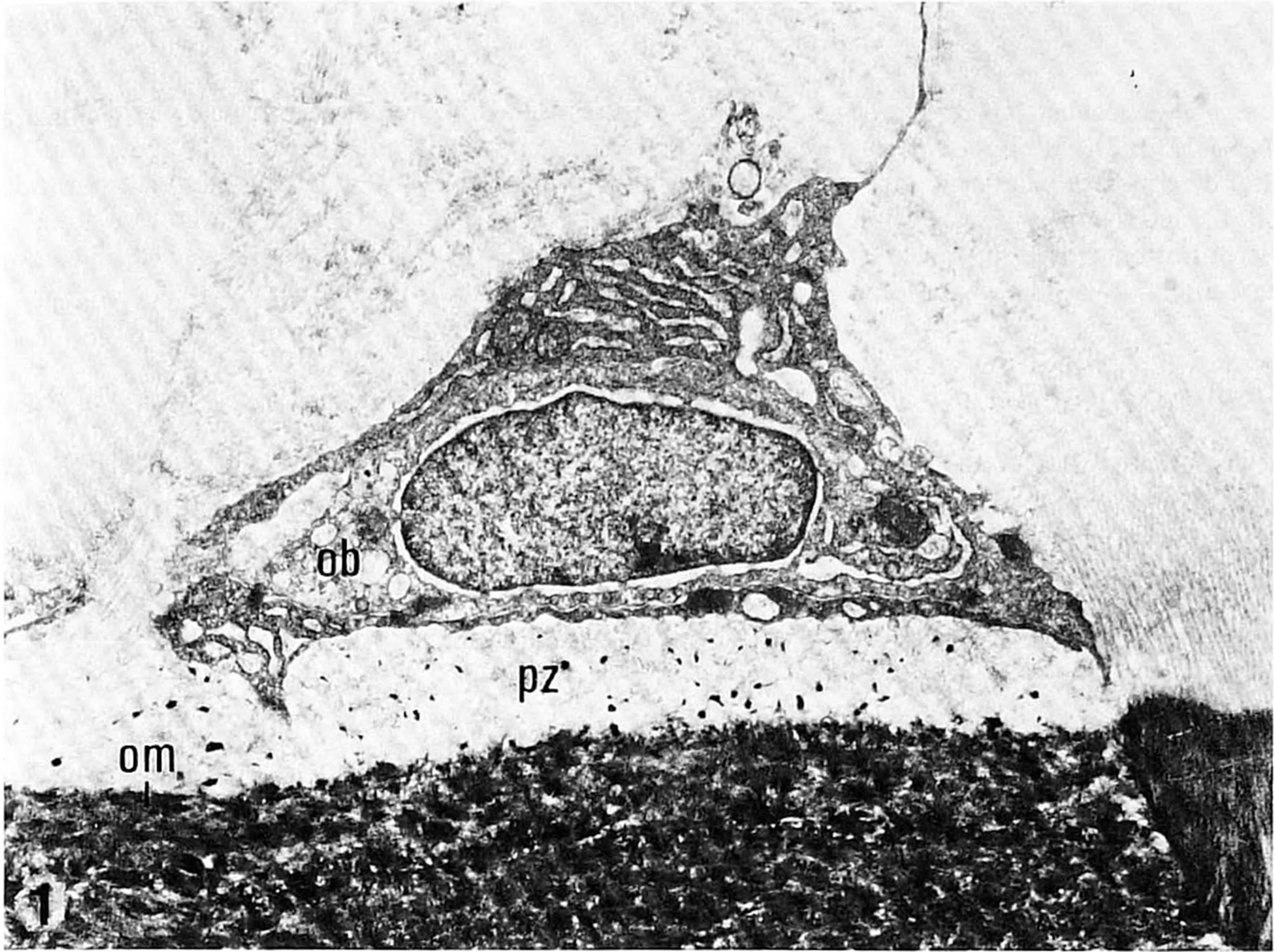
*Vitamin D metabolites.* 1,25(OH)<sub>2</sub>D<sub>3</sub> (Rocaltrol) and 24R,25(OH)<sub>2</sub>D<sub>3</sub> were kindly provided by Hoffmann-LaRoche Holland through Dr. P.J. Pijper. The metabolites were injected intraperitoneally in daily doses in 75 µl sesame oil of 0.1, 1.0 or 10 ng/g for 1,25(OH)<sub>2</sub>D<sub>3</sub> and of 2, 20 and 200 ng/g for 24R,25(OH)<sub>2</sub>D<sub>3</sub>. Injections were given 72 h, 48 h and 24 h before killing of the fish. In an additional experiment the fish received 7 daily injections with either 1 ng/g/day 1,25(OH)<sub>2</sub>D<sub>3</sub> or 20 ng/g/day 24,25(OH)<sub>2</sub>D<sub>3</sub>. All controls were injected with sesame oil.

*Light and electron microscopy.* After anesthetization the fish were decapitated and parts of the tail fins dissected and prefixed for 15 min in 3% glutaraldehyde in 0.1 N cacodylate buffer at 20°C. Fixation followed for 1 h in a similarly buffered and freshly prepared mixture of 2% OsO<sub>4</sub>, 3% glutaraldehyde and 5% K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> (1:1:1), at 0°C. The tissues were poststained in 1% uranyl acetate for 1 h and, after dehydration in ethanol, embedded in Spurr's resin.

For light microscopic purposes 1 µm thick sections were cut with glass knives and stained with toluidine blue. Cellular and nuclear areas were determined in cross sections of fin rays, after projection of the light microscopic images on a Kontron Digiplan magnetostriction tablet. Per fish 50 cells and their nuclei were measured. The number of osteoblasts was estimated in cross sections by determining the number of cells apposing the bone surfaces over a length of 100 µm per fish. Only cell profiles showing nuclei were considered.

For electron microscopy ultrathin sections were cut with diamond knives and stained with Reynolds' lead citrate.

The quantitative data were analysed for statistical significance with Student's *t*-test (two-sided).



**Figs. 1-3.** Periosteal area of fin rays; *ob* osteoblast; *pz* pre-osseous zone; *om* osseous matrix

**Fig. 1.** Control.  $\times 12000$

**Fig. 2.**  $1,25(\text{OH})_2\text{D}_3$ ; 1 ng/g/day for 3 days; *ger* distended granular endoplasmic reticulum; *sg* secretory globule.  $\times 12000$

**Fig. 3.**  $1,25(\text{OH})_2\text{D}_3$ , 1 ng/g/day for 3 days; the osteoblast shows a nucleus with condensed chromatin, and in the electron-dense cytoplasm granular endoplasmic reticulum is almost absent.  $\times 9000$

**Bone mineral content.** Opercular bones and fin rays were cleaned from adhering tissue by immersion in 1 N KOH for 2 h. The bone elements were rinsed three times in distilled water and dried for 16 h at 90° C. After determination of dry weight, the bones were dissolved in 10 N HNO<sub>3</sub> for 2 h. After neutralization with 1 N KOH and appropriate dilution with distilled water the calcium concentrations were measured by microtitration with EGTA in a Marius Calcium Titrator. Phosphate concentrations were determined by the 1-amino-2-naphthol-4-sulfonic acid procedure in a Technicon Autoanalyser.

## Results

The general structure of the fin rays of *S. mossambicus* has been described by Lanzing (1976) and Wendelaar Bonga and Lammers (1982). In cross sections of the tail fins, the fin rays appear as semilunar hemisegments, covered with a thin periosteal layer and surrounded by connective tissue. Three zones can be distinguished: a central, mineralized osseous zone, a hardly-mineralized pre-osseous matrix at the perimeter of the osseous zone, and a single layer of bone-forming cells (osteoblasts). The bone of *S. mossambicus* is of the acellular type: it consists of compact bone without lacunar spaces, canaliculi or encapsulated osteocytes (Fig. 1).

**Controls.** In the slowly growing fish used in this study, the osteoblasts form a single discontinuous layer closely adherent to the preosseous matrix which consists of a narrow zone of densely packed collagen fibers. Mineralization in this zone is restricted to some dispersed apatite crystals (Fig. 1). The osteoblasts contain nuclei with rather condensed chromatin. The cytoplasm shows some strands and cisterns of granular endoplasmic reticulum and a few mitochondria. Occasionally small clear vesicles are present, mainly along the outer cell membrane of the osteoblastic cells. These vesicles are likely involved in the transport and exocytosis of secretory material that will form the pre-osseous matrix (Lopez et al. 1978; Wendelaar Bonga and Lammers 1982). Evidence for the presence of bone-resorbing cells was not observed.

**1,25(OH)<sub>2</sub>D<sub>3</sub>.** Three daily injections of 1,25(OH)<sub>2</sub>D<sub>3</sub> at a low dose (0.1 ng/g/day) did not noticeably influence fin ray structure. However, at 1 ng/g/day this metabolite induced marked changes in the ultrastructure of the osteoblasts. Cell

**Table 1.** Cell and nuclear area, and number per unit length of bone surface, of osteoblasts in slowly growing fish<sup>a</sup>

	Cell area (µm <sup>2</sup> )	Nuclear area (µm <sup>2</sup> )	No. of osteoblasts (cells/nm)
Controls	15.8 ± 3.1	5.9 ± 0.6	59.0 ± 8.3
1,25(OH) <sub>2</sub> D <sub>3</sub> <sup>b</sup>	10.2 ± 2.7*	4.2 ± 0.5**	40.1 ± 7.2*
24,25(OH) <sub>2</sub> D <sub>3</sub> <sup>c</sup>	29.6 ± 5.4**	7.4 ± 0.7**	86.4 ± 14.0**

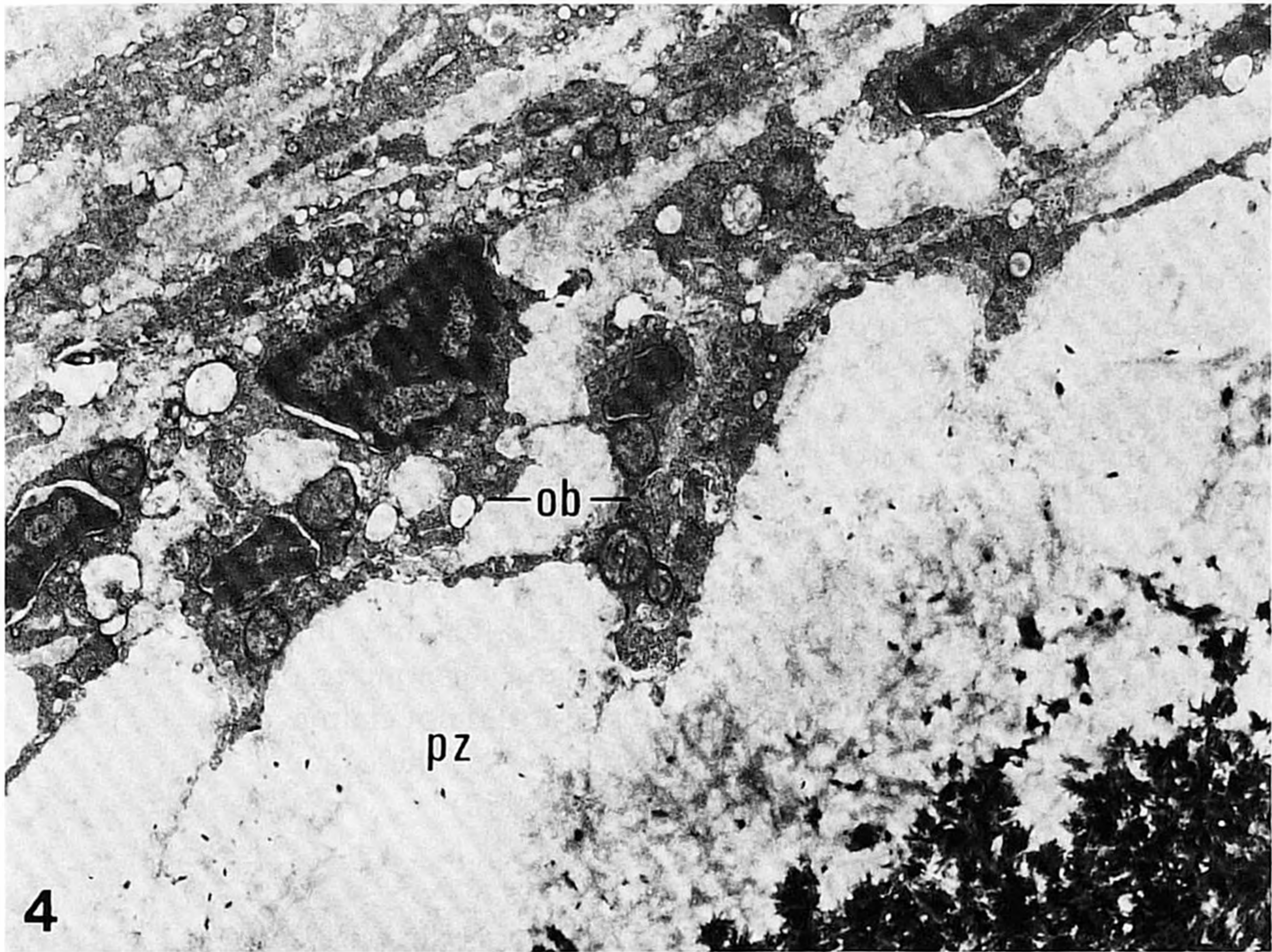
<sup>a</sup> Data given as means ± S.D. of 6 fish per group

<sup>b</sup> Three daily injections of 1 ng/g/day

<sup>c</sup> Three daily injections of 20 ng/g/day

\* Significantly different from controls, *P* < 0.05

\*\* Significantly different from controls, *p* < 0.01



Figs. 4, 5. Periosteal area of fin rays; *ob* osteoblast; *pz* pre-osseous zone

**Fig. 4.**  $1,25(\text{OH})_2\text{D}_3$ , 10 ng/g/day for 3 days; osteoblasts show nuclei with condensed chromatin, and in the electron-dense cytoplasm cellular organelles are scarce.  $\times 11\,300$

**Fig. 5.**  $24,25(\text{OH})_2\text{D}_3$ , 20 ng/g/day for 3 days; the osteoblasts show large nuclei and the cytoplasm is well developed, with arrays of granular endoplasmic reticulum and many small mitochondria.  $\times 12\,000$

and nuclear areas decreased significantly ( $P < 0.05$  and  $P < 0.01$ , respectively; Table 1). The nuclear chromatin became more condensed. In some cells the cytoplasm was reduced to small rims around the nuclei (Fig. 3). In other cells, in which the cytoplasm was less reduced, the membranes of the granular endoplasmic reticulum were distended, probably by the storage of presecretory substances. Occasionally large secretory globules were found, which further points to increased storage and reduced release of secretory material (Fig. 2). The number of cells per unit length of bone surface decreased significantly (Table 1). Injections of higher doses (10 ng/g/day) of  $1,25(\text{OH})_2\text{D}_3$  for three days led to an even more pronounced involution of the osteoblasts (Fig. 4).

After 7 daily injections of  $1,25(\text{OH})_2\text{D}_3$  (1 ng/g/day) the osteoblasts had decreased further in size and number. Large areas of the pre-osseous zone were no longer bordered by bone cells. However, neither after three injections nor after 7 injections indications were found for the presence of bone-resorbing cells.

The bone mineral content of the osseous zone did not change noticeably after 3 daily injections. After injection for 7 days, however, the calcium and phosphate concentrations were significantly decreased (Table 2).

$24,25(\text{OH})_2\text{D}_3$ . Three daily injections (2 ng/g/day) of  $24,25(\text{OH})_2\text{D}_3$  did not noticeably affect bone structure. However, a higher dose (20 ng/g/day for three days) had marked effects on osteoblast structure and numbers: these cells were apparently activated by this metabolite. Cells and nuclei were significantly enlarged (Table 1). The nuclear chromatin was less electron dense than in the controls and prominent nucleoli, hardly found in the controls, were visible in some nuclei (Fig. 5). The cytoplasm contained extensive granular endoplasmic reticulum (Figs. 5, 6) and occasionally a small Golgi area was observed. Clear vesicles were frequently found along the outer cell membranes (Fig. 6). The number of

**Table 2.** Calcium and phosphate contents (millimoles per gram dry weight) in opercular bone and fin rays of slowly growing fish<sup>a</sup>

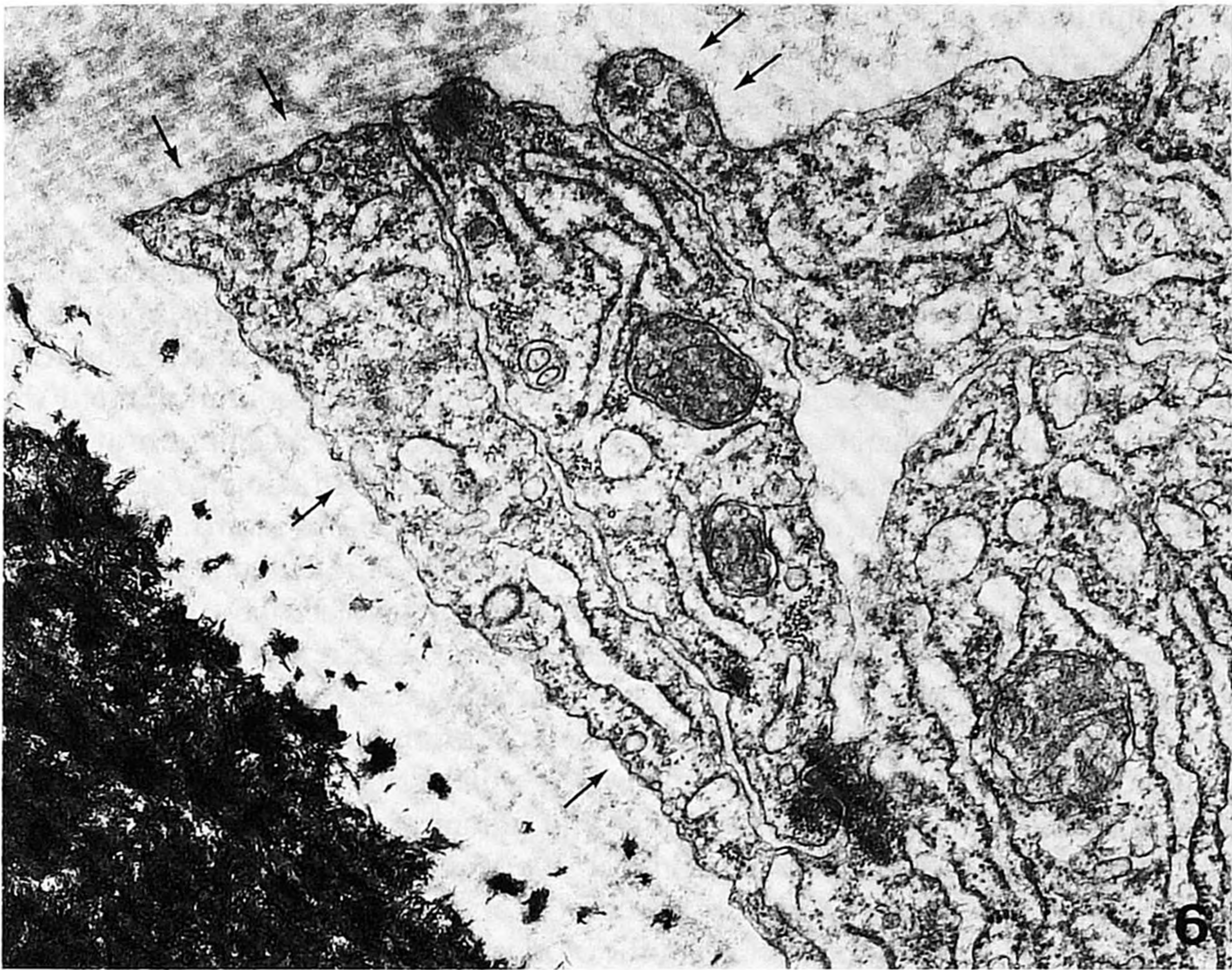
	Operculum		Fin rays	
	Calcium	Phosphate	Calcium	Phosphate
Controls				
3 days	5.88 ± 0.31	3.48 ± 0.19	5.68 ± 0.27	3.39 ± 0.17
7 days	5.84 ± 0.22	3.49 ± 0.14	5.72 ± 0.25	3.40 ± 0.12
$1,25(\text{OH})_2\text{D}_3$ <sup>b</sup>				
3 days	5.88 ± 0.23	3.43 ± 0.16	5.70 ± 0.29	3.45 ± 0.18
7 days	5.31 ± 0.25*	3.18 ± 0.17	5.13 ± 0.26*	3.11 ± 0.14*
$24,25(\text{OH})_2\text{D}_3$ <sup>c</sup>				
3 days	5.77 ± 0.31	3.50 ± 0.26	5.79 ± 0.32	3.53 ± 0.13
7 days	5.91 ± 0.32	3.55 ± 0.17	5.92 ± 0.27	3.57 ± 0.14

<sup>a</sup> Data given as means ± S.D. of 6 fish per group

<sup>b</sup> 3 or 7 daily injections, 1 ng/g/day

<sup>c</sup> 3 or 7 daily injections, 20 ng/g/day

\* Significantly different from controls,  $p < 0.05$



**Fig. 6.** 24,25(OH)<sub>2</sub>D<sub>3</sub>, 20 ng/g/day for 3 days; part of osteoblasts showing extensive granular endoplasmic reticulum and some peripherally located small clear vesicles (*arrows*). × 30 000

osteoblasts per unit area of bone surface had increased significantly (Table 1). Whereas in the controls the layer osteoblasts is discontinuous, in the 24,25(OH)<sub>2</sub>D<sub>3</sub> treated fish the bone cells formed a closed layer that completely covered the skeletal elements.

The vitamin D<sub>3</sub> metabolite did not noticeably affect the degree of mineralization of the bone, neither if administered for 3 days nor for 7 days (Table 2).

High doses of 24,25(OH)<sub>2</sub>D<sub>3</sub> (200 ng/g/day for 3 days) had essentially the same effect as 20 ng/g/day.

## Discussion

Our results show that 1,25(OH)<sub>2</sub>D<sub>3</sub> as well as 24,25(OH)<sub>2</sub>D<sub>3</sub> have distinct but antagonistic effects on bone metabolism in *S. mossambicus*. Whereas 1,25(OH)<sub>2</sub>D<sub>3</sub> has an inhibitory action on osteoblast activity and a slight demineralizing effect on bone matrix, 24,25(OH)<sub>2</sub>D<sub>3</sub> clearly stimulates osteoblast activity.

To some extent the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on bone in *S. mossambicus* are comparable with those described for mammals, birds and the only other teleost fish studied in this respect, the European eel. In rats and birds 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulates PTH secretion (Canterbury et al. 1978; Henry et al. 1977). Since PTH inhibits osteoblast activity the vitamin D<sub>3</sub> metabolite will affect the osteoblasts indirectly. A



direct inhibitory activity of  $1,25(\text{OH})_2\text{D}_3$  was observed in studies on isolated osteoblasts (Cohn and Wong 1978). The demineralizing effect we observed after prolonged injection of  $1,25(\text{OH})_2\text{D}_3$  in *S. mossambicus* resembles the action of this metabolite on bone of mammals, birds and eels. In mammals and birds  $1,25(\text{OH})_2\text{D}_3$  is considered the biologically most active form of vitamin  $\text{D}_3$  in mobilizing calcium and phosphate from bone. This demineralization takes place through the action of osteoblasts and encapsulated osteocytes (Barnes and Lawson 1978; Cohn and Wong 1978). In eels,  $1,25(\text{OH})_2\text{D}_3$  has also been reported to stimulate osteoclastic resorption and osteocytic osteolysis and to reduce the mineral content of the bone (Lopez et al. 1977). However, this metabolite inhibited the exceptionally high osteoclastic activity that accompanies sexual maturation in female eels, and stimulated osteoblastic activity in these fish (Lopez et al. 1980). This effect is reminiscent of the capacity of  $1,25(\text{OH})_2\text{D}_3$  to heal rachitic bone lesions in mammals and birds (Barnes and Lawson 1978; Malluche et al. 1980).

The cells involved in the demineralizing action of  $1,25(\text{OH})_2\text{D}_3$  in mammals, birds and eels (osteoclasts and encapsulated osteocytes) are both lacking in the acellular bone we studied. We have observed multinuclear osteoblasts only in the skull bones, that are undergoing rapid remodeling during growth, but not in fin rays or opercular bones (unpublished results). Mononuclear bone resorbing cells have also not been observed (Wendelaar Bonga and Lammers 1982). Thus, it is unlikely that the demineralization observed in *S. mossambicus* bone after prolonged  $1,25(\text{OH})_2\text{D}_3$  treatment is effected through the action of bone-resorbing cells. It may be a consequence of a reduction of the blood calcium levels in these fish. In the course of this study we occasionally observed indications for a hypocalcemic effect of  $1,25(\text{OH})_2\text{D}_3$  (unpublished observations). This hypocalcemia contrasts with the hypercalcemic effect that may accompany  $1,25(\text{OH})_2\text{D}_3$  treatment in higher vertebrates (Rasmussen and Bordier 1980).

Administration of  $24,25(\text{OH})_2\text{D}_3$  clearly activates the osteoblastic cells in *S. mossambicus*, at doses in the same range as have been used for higher vertebrates. In mammals and birds  $24,25(\text{OH})_2\text{D}_3$  has also been associated with bone formation, and it has been considered a calcium-stimulating hormone of physiological importance (Kanis et al. 1978; Rasmussen and Bordier 1980), with effects different from, and often opposing, those of  $1,25(\text{OH})_2\text{D}_3$ . Whereas the latter metabolite stimulates PTH secretion,  $24,25(\text{OH})_2\text{D}_3$  causes a fall in plasma PTH levels (Canterbury et al. 1978). This effect indicates that  $24,25(\text{OH})_2\text{D}_3$  inhibits indirectly the mobilization of calcium and phosphate from bone, in contrast to  $1,25(\text{OH})_2\text{D}_3$ , which stimulates this process. Ornoy et al. (1978) concluded that  $24,25(\text{OH})_2\text{D}_3$  is required for normal bone formation in chicks, and  $24,25(\text{OH})_2\text{D}_3$ , but not  $1,25(\text{OH})_2\text{D}_3$ , may stimulate biosynthesis and sulfation of glycosaminoglycans by isolated rat chondrocytes (Corvol et al. 1978).

The biological significance of  $24,25(\text{OH})_2\text{D}_3$  has been questioned in a series of studies on vitamin  $\text{D}_3$  analogs. Hydroxylation is important for the physiological functions of vitamin  $\text{D}_3$ . When hydroxylation of  $25(\text{OH})\text{D}_3$  at the 24-position was blocked with two fluorine atoms, the compound formed ( $24,24$ -difluoro- $25(\text{OH})\text{D}_3$ ) appeared to be equivalent to  $25(\text{OH})\text{D}_3$ , and only slightly less active than  $1,25(\text{OH})_2\text{D}_3$ , in stimulating intestinal calcium transport, mobilization of calcium from bone, elevation of serum calcium and phosphate, or calcification of rachitic bone in vitamin  $\text{D}_3$ -deficient rats (Tanaka et al. 1979; Halloran et al. 1981;

Kabakoff et al. 1982). These effects are understandable since the di-fluoro-compound, like 25(OH)D<sub>3</sub> but unlike 24,25(OH)<sub>2</sub>D<sub>3</sub>, is rapidly oxidized at the 1-position (Boyle et al. 1973), and then it has affinity for the same receptors as 1,25(OH)<sub>2</sub>D<sub>3</sub> (Tanaka et al. 1979). The results with the difluoro-analogue therefore indicate that blocking of the 24-position does not notably affect the actions of 25(OH)D<sub>3</sub> that require 1-hydroxylation. They further show that vitamin D<sub>3</sub> metabolites that are not hydroxylated at the 24-position may promote bone growth and mineralization in rachitic rats (Tanaka et al. 1979; Halloran et al. 1981). However, these results do not exclude the possibility that 24-hydroxylation of 25(OH)D<sub>3</sub> may lead to a compound that has specific effects on bone formation distinct from those of the 1-hydroxylated metabolites. Whereas 24,25(OH)<sub>2</sub>D<sub>3</sub> may have little biological activity in bioassays typical for 1,25(OH)<sub>2</sub>D<sub>3</sub> in chicks (Holick et al. 1976), more recently Ornoy et al. (1978) and Malluche et al. (1980) have demonstrated that both 1,25- and 24,25(OH)<sub>2</sub>D<sub>3</sub> are required for normal bone development and healing of rachitic bone lesions in chicks. Henry and Norman (1978) showed that normal egg development occurred only when vitamin D-deficient hens received a combination of 1,25- and 24,25(OH)<sub>2</sub>D<sub>3</sub>. These results further indicate that both metabolites have different actions on bone.

Thus, the possibility should be taken into consideration that 24,25(OH)<sub>2</sub>D<sub>3</sub>, like 1,25(OH)<sub>2</sub>D<sub>3</sub>, is a hormonally active metabolite of vitamin D<sub>3</sub> in mammals, in birds and, as indicated by the present study, in fish. Our observations on fish suggest that, from an evolutionary point of view, 1,25(OH)<sub>2</sub>D<sub>3</sub> as well as 24,25(OH)<sub>2</sub>D<sub>3</sub> may have a long history as distinct endocrine factors.

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