

Parathyroid Hormone-Like Effects of Rainbow Trout Stannius Products on Bone Resorption of Embryonic Mouse Calvaria *in Vitro**

FLORIS P. J. G. LAFEVER, H. I. M. B. SCHAEFER, M. P. M. HERRMANN-ERLEE,
AND S. E. WENDELAAR BONGA

Department of Zoology II (F.P.J.G.L., H.I.M.B.S., S.E.W.B.), University of Nijmegen, 6525 ED Nijmegen, The Netherlands; and Laboratory for Cell Biology and Histology (F.P.J.G.L., M.P.M.H.-E.), University of Leiden, 2333 AA Leiden, The Netherlands

ABSTRACT. Products of the Stannius corpuscles (SC) of rainbow trout were tested in an established PTH bioassay involving bone resorption in embryonic mouse calvaria. Aqueous extracts from Stannius corpuscles (SC-homogenate) showed a bone-resorbing activity comparable to PTH in 24-h cultures of calvaria, indicated by a dose-dependent stimulation of lactate production and of calcium, phosphate as well as β -glucuronidase release. Moreover, SC-homogenates induced an increase in os-

teoclastic activity. The PTH-like SC-principle is released during *in vitro* incubations of the glands. These results and the lack of an additive effect of SC-products and PTH on bone resorption suggest that both products activate the same receptor. We hypothesize that the hypocalcemic hormone of the SC of fish shares structural resemblance with PTH, the major hypercalcemic hormone of terrestrial vertebrates. (*Endocrinology* 119: 2249-2255, 1986)

STANNIUS corpuscles (SC) are small endocrine glands characteristic of holostean and teleostean fish. Rainbow trout usually have two to five ovoid corpuscles, located ventrocaudally to the kidney, that vary in diameter from 2 to 3 mm (1). For several species it has been reported that two different endocrine cell types are present (1-5). There is ample evidence that the predominant cell type produces a hypocalcemic factor, whereas the second cell type may be involved in sodium and potassium regulation (6, 7). Effects of SC-products on plasma phosphate, magnesium, and chloride have been reported, but these effects are usually rather small and may result from a disturbance of calcium metabolism (6, 7). Plasma calcium concentrations rise considerably after stanniectomy (STX); SC implants or injections of SC-extracts completely reverse STX-induced hypercalcemia (8, 9).

Structural resemblance has been suggested between the hypocalcemic factor of the SC and PTH, a hormone considered typical for terrestrial vertebrates and absent in fish. Antibodies raised against bovine PTH (bPTH)

(1-84) showed cross-reactivity with a substance in eel blood plasma. This substance disappeared from the blood after STX and increased after the infusion of calcium chloride (10). Also, secretory substances of the SC cells have been shown to cross-react with antibodies raised against bPTH. Overloading the blood with calcium chloride resulted in the release of anti-bPTH positive secretory material (11).

The immunological similarities between the SC principle and PTH prompted us to investigate a possible relationship in bioactivity between PTH and the SC principle.

In higher vertebrates PTH is known to stimulate bone resorption. For rodents the effects of pharmacological doses of PTH on several parameters for bone resorption *in vitro* have been thoroughly investigated. Typically, PTH-induced bone resorption is accompanied by an increased lactate production, as a result of enhanced aerobic glycolysis (12). PTH-induced bone resorption results from an increase in the number of osteoclasts as well as in their bone-resorbing activity (13). Increased osteoclastic activity and increased release of lysosomal enzymes [*e.g.* β -glucuronidase (14)] as well as the removal of bone mineral components [mainly calcium and phosphate (12)] may serve, therefore, as parameters for PTH-induced bone resorption.

We used embryonic mouse calvaria, a tissue with con-

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Address correspondence and requests for reprints to: F. Lafever, Department of Zoology II, University of Nijmegen, Toernooiveld 25, 6525 ED Nijmegen, The Netherlands.

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siderable PTH-sensitive bone-resorbing activity (12), to characterize the bioactivity of the SC principle, using the forementioned parameters.

Materials and Methods

Calvarium culture technique

Calvaria were removed from 18-day-old mouse embryos and each calvarium was bisected. The left half of one calvarium and the right half of the second calvarium and *vice versa* were fixed at 2 cm from the bottom in a 20-ml roller tube, containing 1 ml culture medium. The tubes were placed in an almost horizontal position in a roller drum for 24 h (six to seven rotations per h) (15). The culture medium consisted of 90% Hanks' balanced salt solution (Hanks' BSS) and 10% heat inactivated human serum. Total calcium and phosphate concentrations in this medium were 2.7 and 1.8 mM, respectively. After 24 h of incubation at 37 C, calvaria were removed from the incubation medium that was analyzed within 24 h for calcium, phosphate, lactate, and β -glucuronidase activity. Media were stored, frozen at -20 C; the activity of β -glucuronidase has been reported not to decrease over a 24-h period (16).

Hormone administration

SC homogenate. Stannius corpuscles (a generous gift from Dr. N. Mayer-Gostan; Laboratoire Jean Maetz; Villefranche-sur-Mer, France) were collected from rainbow trout (*Salmo gairdneri*); whole glands were lyophilized and stored at -60 C. Approximately 25 mg lyophilized material (equivalent to about 100 mg wet weight SC, the amount of tissue obtained from 10 kg trout) were homogenized in 2.5 ml ice-cold Hanks' BSS. After centrifugation (15 min; at $9000 \times g$) the supernatant was diluted to desired concentrations with culture medium, and used within 2 h. Doses were expressed in milligrams of wet weight of homogenized SC per ml calvarium culture medium.

Collection of SC-products released in vitro. After a minimum acclimation period of 2 weeks to Nijmegen tapwater (0.8 mM Ca), rainbow trout were killed, and the SC removed, weighed, and rinsed in Hanks' BSS. Subsequently they were incubated for 5 h at 22 C in Hanks' BSS in a shaking water-bath (~10 mg wet weight/150 μ l Hanks' BSS). The incubations were terminated by removal of the corpuscles. The media were quickly frozen in liquid N₂ and stored at -60 C, for a maximum of 7 days. These media were thawed shortly before use and diluted to desired concentrations with culture medium. In these experiments only the calcium concentration of the medium was determined as a measure for bone resorption and an experimental set-up was used that allowed paired observations as described elsewhere (15). SC doses were expressed as milligrams of wet weight, used for incubation, per ml calvarium culture medium.

PTH. PTH was purchased from Sigma (St. Louis, MO; trichloroacetic acid powder; Cat. no. p0892; 140 U/mg) and dissolved in 0.005 N acetic acid containing 1% Pentex-albumin. Solutions of 1 IU/ μ l were stored in liquid N₂. For the parameters tested in this study no differences were observed between the effects

of this purified PTH and synthetic bPTH(1-34) (12). Immediately before use, PTH was diluted to desired concentrations with culture medium. Doses are expressed as international units of PTH per ml calvarium culture medium.

Controls. SC-homogenates were heat-treated by a 15-min incubation at 100 C. Bone-resorbing activity of such heat-treated homogenates was tested using forementioned biochemical parameters. In another set of experiments 100-mg tissue samples (wet weight) of rainbow trout liver, brain, muscle, and trunk kidney were removed and homogenized in 1 ml ammonium acetate (50 mM, pH 7.4). After centrifugation (5 min, $9000 \times g$) supernatants were lyophilized and stored at -20 C. For experiments the samples were dissolved in culture medium to a concentration of 5 mg wet weight SC/ml culture medium. Release of calcium and phosphate was determined as a measure for bone resorption.

Analytical methods

Medium total calcium content was determined with a commercial colorimetric calcium-kit (Sigma Cat. no. 586). Inorganic phosphate was measured according to the method of Delsal and Manhourin (17). Combined calcium phosphate standards (Sigma Cat., no. 360-11) were used as reference. The medium lactate concentration was measured as described by Lowry *et al.* (18), using an autoanalyzer method (19). Lithium lactate (Sigma) was used as reference. The activity of β -glucuronidase in the culture medium was determined according to Mead *et al.* (20), using the conversion of 4-methyl-umbelliferyl-glucuronide (Boehringer-Mannheim GmbH, Mannheim, W. Germany) to 4-methyl-umbelliferyl during a 30-min incubation period at 37 C; 4-methyl-umbelliferyl was used as a reference.

Histology

For light microscope examinations, calvaria were incubated as described above. After a 24-h incubation period, calvaria were fixed in phosphate buffered (pH 7.0) 4% glutaraldehyde solution (24 h at 4 C) and decalcified in 5% formic acid, containing 5% formaldehyde (3 h at 4 C), followed by two washes in distilled water. Subsequently, acid-phosphate staining was performed according to Barka and Anderson (21). Calvaria were embedded in paraffin; 5- μ m sections were stained with acid hematoxylin. Serial sections from calvarium halves were used to quantify osteoclast nuclei; in every fourth section nuclei surrounded by acid-phosphatase positive cytoplasm were scored. Results are expressed as the mean number of osteoclast nuclei per section \pm SEM for three calvarium halves.

Statistical analysis

Statistical evaluation was performed by the use of the Mann-Whitney *U* test (one-tailed). For paired observations Student's *t* test was used. Significance was accepted at $P < 0.05$. Mean values \pm SEM are given.

Results

Biochemical observations

Lactate production. Figure 1 shows the relationship between the increase in lactate production by calvaria and

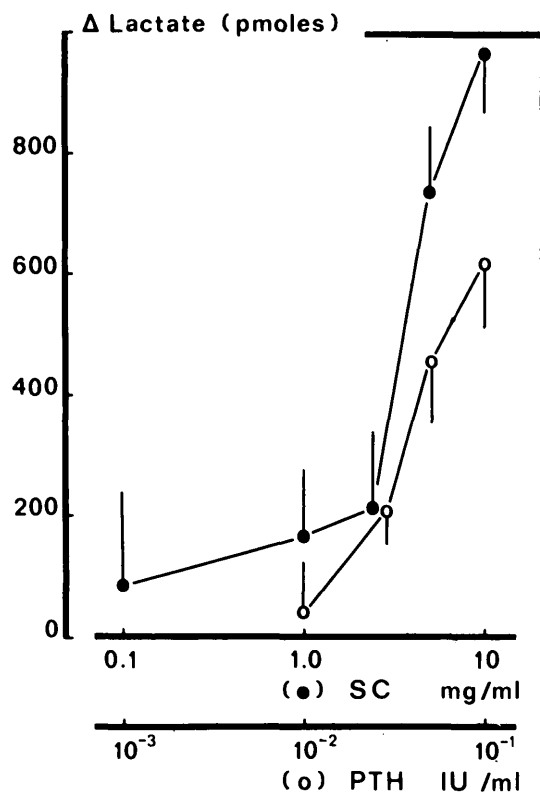


FIG. 1. Effects of SC-homogenate (●—●) and PTH (○—○) on lactate production by two calvarium halves, cultured for 24 h. Mean values \pm SEM are given; for SC-homogenate $n = 16-24$ and for PTH $n = 4-16$. Significant stimulation of lactate production occurred at concentrations above 2.5 mg/ml SC-homogenate or 10^{-2} IU/ml PTH.

the concentrations of SC-homogenate or PTH. SC-homogenate and PTH yielded a similar activation pattern. Proceeding from an equipotent bioactivity of 10 mg SC tissue and 0.1 IU PTH, no significant difference between the degree of stimulation by SC-homogenate or by PTH could be observed. At the concentration of SC-homogenate tested no maximum lactate production was observed. This observation corroborates data that we obtained for the effects of PTH on embryonic mouse calvaria. As much as 1 IU PTH/ml is needed to obtain a maximum lactate production (12). Proceeding from a similar pattern for PTH and for SC-homogenate-induced lactate production, it may be calculated that at least 100 mg SC-homogenate/ml medium would be required to produce maximum lactate production.

Heat treatment of SC-homogenates significantly diminished lactate production, although the stimulatory effect on lactate production by SC-homogenates was not completely abolished (Fig. 2).

Bone demineralization. Figure 3 shows a dose-response curve for SC-homogenate and PTH-induced calcium release from embryonic mouse calvaria. An increase in the amount of SC-product resulted in an increase of calcium resorption from the bone. As shown in Fig. 4, a

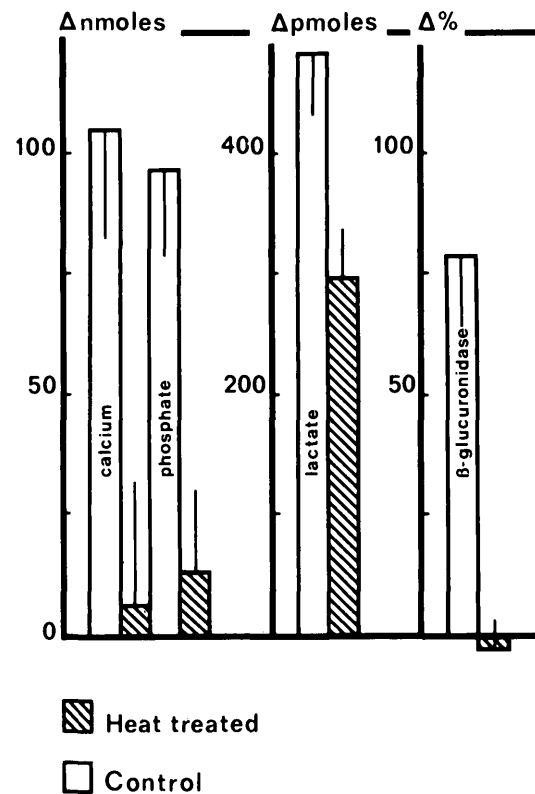


FIG. 2. Lactate production, calcium, phosphate, and β -glucuronidase release induced by untreated and heat-inactivated SC-homogenate (10 mg/ml), during a 24-h incubation period. Mean values \pm SEM are given; $n = 8$; all differences with the controls are statistically significant.

similar dose-response relationship was found for SC-homogenate and phosphate release from calvaria. Using calcium and phosphate release as parameters for bone resorption, again 10 mg SC wet weight could be equated with about 0.1 IU PTH. The maximum amounts of calcium and phosphate, released by SC-homogenate after a 24-h cultivation period, were similar to the values observed for the maximum release induced by PTH. Half-maximum calcium and phosphate release occurred at about 2.5 mg SC wet weight/ml, and at about 2.5×10^{-2} IU/ml PTH. The value for PTH is in good agreement with data from the literature (12).

Maximum stimulation of calcium and phosphate release was obtained by the addition of 0.1 IU PTH or 10 mg wet weight SC-homogenate separately. No further increase in calcium and phosphate release could be observed when maximally stimulating doses of SC-homogenates and PTH were added together (Table 1).

Heat treatment of SC-homogenates resulted in a complete loss of the ability to demineralize the calvaria (Fig. 2).

Bone resorption in mouse calvaria was also induced by the products that are released during *in vitro* incubation of SC. In Fig. 5 the dose-response relationship for SC secretory products and calcium release is shown. A sim-

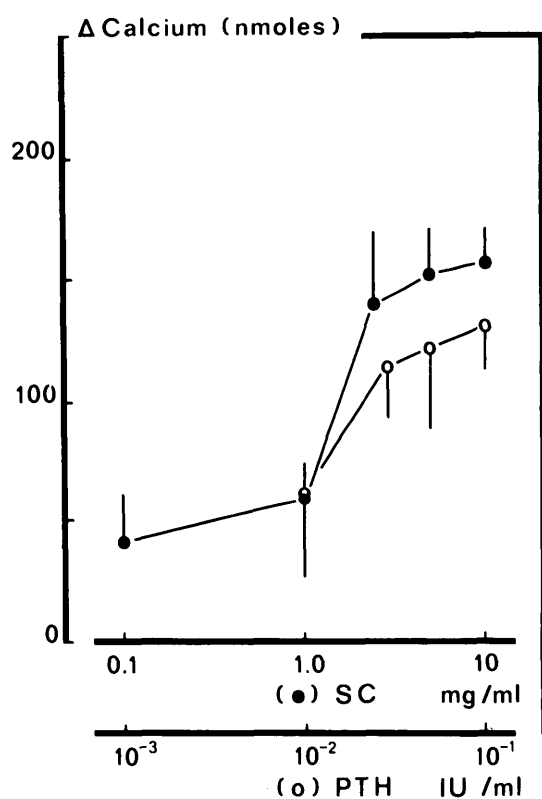


FIG. 3. Effects of SC-homogenate (●—●) and PTH (○—○) on calcium release from two calvarium halves, cultured for 24 h. Mean values \pm SEM are given; for SC-homogenate $n = 16-24$ and for PTH $n = 4-10$. Significant stimulation of calcium release occurred at concentrations above 1.0 mg/ml SC-homogenate and all PTH concentrations tested.

ilar potency to stimulate calcium release was found for SC-homogenates and the products released by the same amount of tissue during a 5-h incubation period.

β -glucuronidase release. Figure 6 shows the dose response relationship for β -glucuronidase release induced by SC-homogenate and PTH. An increasing amount of SC-homogenate resulted in an increasing release of β -glucuronidase. The release of β -glucuronidase was stimulated by PTH over a range of 0.01 to 1 IU/ml. At a concentration of 0.3 IU/ml and 1 IU/ml we found $83 \pm 22\%$ and $62 \pm 10\%$, respectively (data not shown in Fig. 6). No dose dependency was observed for PTH-induced β -glucuronidase release at the concentrations tested. The activation pattern of PTH differed from the SC-homogenate activation pattern.

Heat treatment of the SC-homogenates resulted in a complete loss of the ability to increase the β -glucuronidase release of calvaria (Fig. 2).

Control homogenates. Neither calcium nor phosphate release was significantly stimulated by tissue homogenates of trout liver, brain, muscle, and kidney (Table 2).

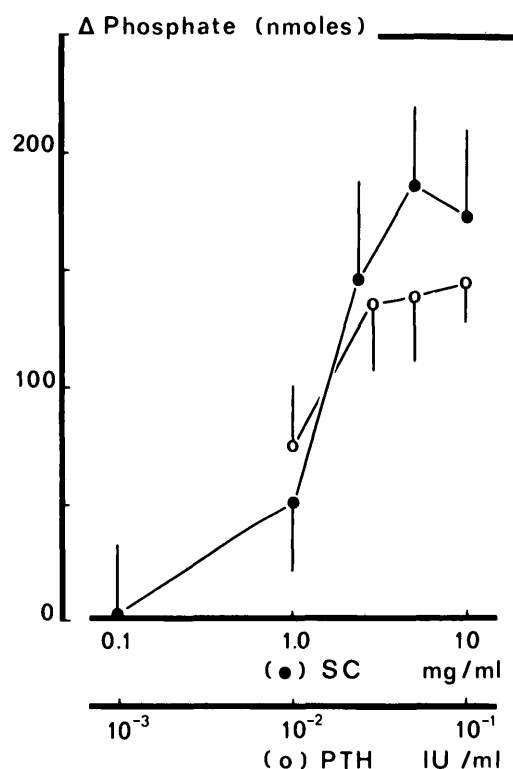


FIG. 4. Effects of SC-homogenate (●—●) and PTH (○—○) on phosphate release from two calvarium halves, cultured for 24 h. Mean values \pm SEM are given; for SC-homogenate $n = 16-24$ and for PTH $n = 4-16$. Significant stimulation of phosphate release occurred at concentrations above 1.0 mg/ml SC-homogenate and all PTH concentrations tested.

TABLE 1. Calcium and phosphate release induced by maximum effect concentrations of SC-homogenate and PTH added separately or together

	Δ Calcium release (nmol)	Δ Phosphate release (nmol)
Stannius (10 mg/ml)	125 ± 44 (6)	119 ± 28 (9)
PTH (10^{-1} IU/ml)	154 ± 33 (9)	91 ± 28 (9)
Stannius + PTH	147 ± 30 (10)	117 ± 16 (10)

Mean values \pm SEM are given with the number of observations in parentheses. No statistically significant differences were observed.

Histological observations

Osteoclast activation. The addition of SC-homogenate as well as of PTH to the incubation medium of the calvaria resulted in a significant increase in the number of osteoclast nuclei after a 24-h incubation period (Table 3). Doses of PTH and SC-homogenate, giving maximum calcium and phosphate release, were tested. At these doses, no significant difference was observed between the effect of SC-homogenate or PTH.

Discussion

We conclude from the present data that the corpuscles of Stannius of rainbow trout produce and secrete a

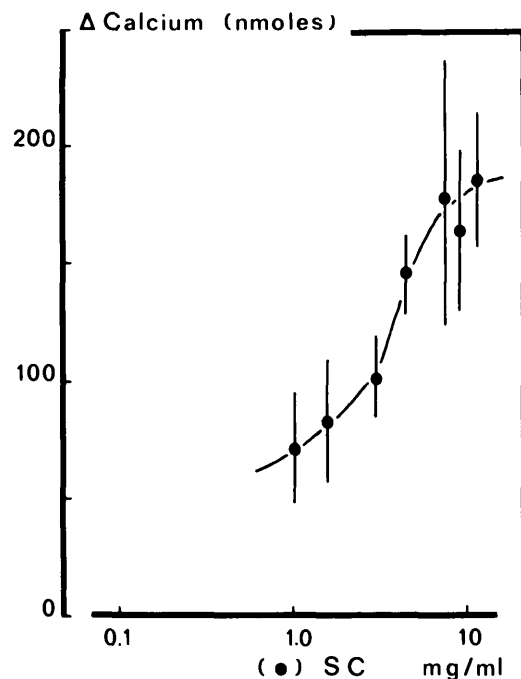


FIG. 5. Calcium release from two calvarium halves cultured for 24 h, induced by SC-products secreted during *in vitro* incubations. Mean values \pm SEM are given; paired observations ($n = 3-6$). Stimulation at concentrations above 1 mg/ml SC (wet weight used for *in vitro* incubation) are statistically different from the release in the absence of hormone.

product that resembles PTH in its bone resorbing action. Aqueous extracts from SC as well as products released by these glands *in vitro* stimulate bone resorption in embryonic mouse calvaria in a way comparable to PTH. Lactate production as well as calcium and phosphate release were stimulated dose dependently. The patterns of lactate production and the release of calcium and phosphate were essentially similar for SC-homogenate and PTH-stimulated samples. In this bioassay 100 mg wet weight SC (the amount of tissue obtained from 10 kg trout) were found to be equivalent to 1 IU PTH. In our assay on the release of β -glucuronidase, however, SC-homogenate proved to be more stimulatory than PTH. The relatively weak and dose-independent stimulatory activity of PTH over this concentration range is not clearly understood. For a comparable bioassay as used in this study, Vaes (14) reported a linear correlation between mineral release and β -glucuronidase release, during 24- to 72-h incubations with 0.01 to 1 IU/ml PTH. We found a comparable relationship between β -glucuronidase and phosphate release after stimulation with SC-homogenate. For PTH, however, such a relationship was not found. Quantitatively, our observations on PTH-induced β -glucuronidase release ($62 \pm 10\%$ increase at 1 IU PTH) compare well with those of Vaes, who showed an increase of 75% after the addition of 1 IU PTH (14).

The specificity of the bone-resorbing effect for the SC-

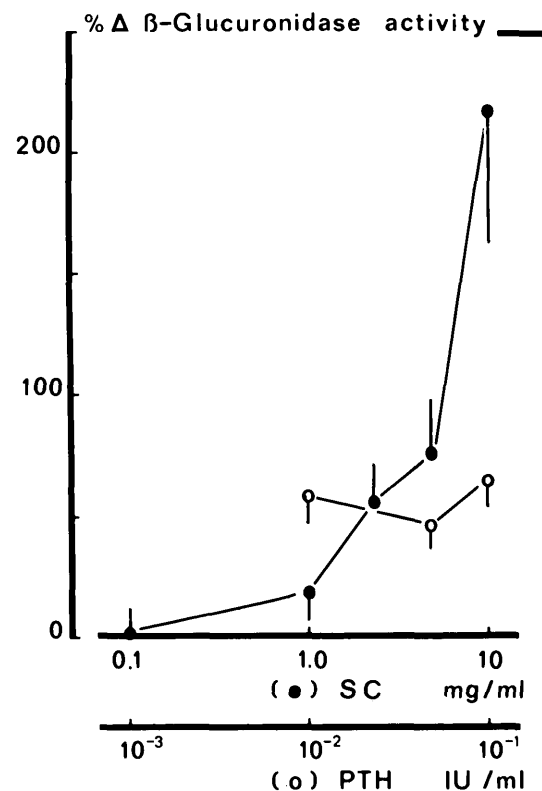


FIG. 6. SC-homogenate and PTH-induced stimulation of the β -glucuronidase activity, during a 24-h culture period. Values \pm SEM are expressed in percent; release in the absence of hormone was equated with 100%. For SC-homogenate $n = 16-24$ and for PTH $n = 4-14$. The increase in the release of β -glucuronidase activity at concentrations above 1.0 mg/ml SC-homogenate, and at all PTH concentrations tested are statistically different from activity in the absence of hormone.

TABLE 2. Calcium and phosphate release induced by tissue homogenates of trout SC, kidney, muscle, brain, and liver (5 mg/ml)

5 mg/ml	Δ Calcium (nmol)	Δ Phosphate (nmol)
Stannius	153 ± 19 (23) ^a	186 ± 37 (24) ^a
Kidney	-22 ± 48 (14)	37 ± 27 (13)
Muscle	-29 ± 25 (11)	8 ± 17 (13)
Brain	-62 ± 14 (9)	-12 ± 15 (13)
Liver	-6 ± 15 (13)	-28 ± 34 (13)

Mean values \pm SEM are given with the number of observations in parentheses.

^a Statistical significance.

homogenates is indicated by the absence of an effect of kidney, liver, brain, and muscle homogenates in this bioassay.

Most likely the PTH-like substance of the Stannius corpuscles is heat labile. Incubation of SC-homogenate at 100 C resulted in a loss of its ability to stimulate the release of calcium, phosphate, and β -glucuronidase. The stimulatory effect of SC-products on lactate production, although significantly diminished by heat treatment, was not reduced to control levels. Apparently, SC-homogenates contain a heat labile factor, involved in bone re-

TABLE 3. Effects of SC-homogenate and PTH on the calvarium osteoclast activity

	No. of nuclei per section (in %)
Control	100 ± 10 (6)
SC-homogenate (5 mg/ml)	158 ± 22 ^a (3)
SC-homogenate (10 mg/ml)	178 ± 31 ^a (3)
PTH (1 IU/ml)	183 ± 1 ^a (2)

The number of nuclei per section of calvarium halves incubated in the absence of hormones is equated with 100%. Mean values ± SEM are given with the number of observations in parentheses.

^a Statistical significance.

sorption as well as a heat stable factor, capable of inducing lactate production but independent from bone resorption. For this bioassay it has been shown before that the lactate production can be stimulated without inducing bone resorption (12).

SC-products increase the number of osteoclast nuclei in mouse calvaria. After an incubation period of 24 h, we observed a similar increase in the number of osteoclast nuclei in the PTH and the SC-homogenate-stimulated calvaria, at concentrations that induce maximum calcium and phosphate release. The method used in our experiments did not allow the determination of the number of osteoclasts or number of nuclei per cell separately. Whether the increase in the number of osteoclast nuclei is the result of an increase in the number of cells or in the number of nuclei per cell remains to be established. Rowe and Hausmann (22), however, have shown that both an increase in the number of nuclei per cell and an increase in the number of osteoclasts (which also results in an increased number of nuclei, but may result from a different stimulatory mechanism) may result in stimulated bone resorption. Our data with respect to PTH corroborate those of Holtrop and co-workers (23) who showed an increase in the number of osteoclasts in rat bones after a 24-h stimulation with PTH. With respect to SC-homogenates, our results corroborate histological observations by Milet *et al.* (24); they reported a stimulation of osteoclastic activity 0.5 h after injections of SC extracts in rat.

The SC principle responsible for bone resorption is released during *in vitro* incubations of Stannius corpuscles. Recent studies in our laboratory give growing evidence for a proteinaceous product, with an apparent molecular mass of approximately 25 kilodaltons, as the putative hormone of these glands (25).

Both PTH and the SC principle appear to exert their bone-resorbing action via the same pathway; no additive effect was observed when maximum stimulating concentrations of PTH and SC-homogenate were tested together. From the close resemblance between PTH and SC-products in the bioassay reported here, we suggest that the Stannius principle stimulates bone resorption

via activation of the PTH receptor.

One could argue that the bone-resorbing effects of SC-homogenates reported in this paper originate from an action by prostaglandins. However the amount of prostaglandins extracted during our homogenization procedure (an aqueous extract) will be very small. More importantly, there is no reason to assume that SC contain more prostaglandins than any of the other control tissues tested.

Fontaine (26, 27) was the first to report, and it is generally accepted now, that the SC produce a factor that is hypocalcemic in fish. More recently, Lopez and co-workers (10, 11) suggested, that this hypocalcemic factor was homologous with PTH. This suggestion was mainly based on immunocross-reactivity of the presumed SC hormone with PTH antisera (10, 11). This immunological resemblance should be considered with caution, however. It has been shown that antisera against PTH cross-react with products present in other endocrine tissues in fish (28). Moreover, not all PTH antisera tested cross-react with SC-products (29), which indicates that the structural similarities between PTH and the SC-product is only partial. On the other hand, in addition to the immunological resemblances and the remarkable similarity in *in vitro* bioactivity reported here, PTH and the putative SC hormone also show similar effects on blood calcium levels *in vivo*. Injections of crude SC-extracts and PTH both elevate blood calcium levels in rats (30), and reduce blood calcium levels in fish (25, 31). All these data strongly suggest that both calcium-regulating hormones have important structural similarities. An attractive hypothesis would be that these structural similarities result from phylogenetically conservative aminoacid sequences in the biologically active parts of PTH and the SC hypocalcemic hormone.

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