Calcif. Tissue Int. 33, 529–540 (1981)

Calcified Tissue International

Biochemical and Histological Studies on Various Bone Cell Preparations

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Summary. Four different cell populations—designated PF, OB, OC, and PC—were isolated from calvaria of 18-day-old chick embryos for analysis of the effects of hormones on bone tissue. The cell populations were studied with histological and biochemical methods. Apart from the well-known cell types present in calvaria, a new cell type was found in the noncalcified organic matrix between the osteoblastic layer and the calcified matrix. These cells were provisionally called osteocytic osteoblasts. They represent the "transition state" between osteoblasts and osteocytes.

On the basis of histological studies with light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM), the PF population was considered to originate primarily from the periosteal fibroblasts, the OB population from the osteoblasts and osteocytic osteoblasts. The population of cells still present in calvaria after removal of periosteal fibroblasts and osteoblasts was called the OC population. This cell population was very much enriched with osteocytes. The fourth isolated population (PC) was a mixed population of fibroblasts, osteoblasts, and preosteoblasts. On exposure to parathyroid hormone (PTH), all four cell populations showed increased lactate production, but only the OB and OC populations displayed increased cAMP production. Prostaglandin E_1 (PGE_1) stimulated cAMP production in both OB and PF cells.

From the results of this study it was concluded that PTH receptors are present on all of the cell types studied, but that occupancy of the receptor induces adenylate cyclase stimulation only in osteocytes and fully differentiated osteoblasts.

Key words: Bone cells — Electron microscopy — PTH — PGE_1.

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During the last few years a vast amount of work has been done on the isolation and cultivation of bone cells. Peck and coworkers [1] were among the first to recognize the possibility of isolating bone cells from calvaria of fetal rats with collagenase and of culturing the isolated cells as a monolayer. This work has been continued and extended by Peck and coworkers [2] and by a number of other groups [3–5]. Recently, a major breakthrough has perhaps been provided by the work of Wong, Luben, and Cohn [6–8]. By repeated treatment of calvaria of newborn mice with collagenase, they obtained two cell populations showing different responses to parathyroid hormone and calcitonin. The results obtained in biochemical studies led them to call these two populations tentatively osteoblast-like and osteoclast-like cells, although there is no morphological proof that these cells are indeed osteoblasts and osteoclasts.

A different method for cell isolation was used by Smith and coworkers [9], who isolated four different cell populations from calvaria and femora of young rats by mechanical means, without the use of digestive enzymes. On the one hand, their method has the advantage of avoiding cellular damage by digestive enzymes, but on the other hand, the resulting cell suspensions are probably less suitable for cell culture because they are prepared from nonembryonic tissues.

In the past we devised yet another method, using cultured periostea of fetal chick calvaria from which the cells were liberated with collagenase [10]. The advantage of this technique was that we obtained a mixed periosteal cell population including osteoblasts, but avoided contamination of the cell suspension with small fragments of mineralized matrix, which in our experience is unavoidable when cells are isolated directly from calvaria. The presence of bone mineral in a cell suspension of course renders the suspension unsuitable for the assessment of calcium transport.

0171-967X/81/0033–0529 $02.40

The drawback of all bone cell isolation procedures used so far is the difficulty of identifying the isolated cells. A detailed histological study was clearly needed. This paper describes the isolation of four different cell populations from fetal chick calvaria by different methods. By careful histological study of the tissue before, during, and after the cell isolations, we attempted to define the different populations as well as possible. In addition, the responses of the different cell types after exposure to parathyroid hormone (PTH), calcitonin (CT), and prostaglandin E_1 (PGE_1) were investigated on the basis of a number of criteria.

Materials and Methods

Cell Isolation

Four different cell populations were prepared. The periosteal cell (PC) population was obtained as described elsewhere [10, 11] from cultured periostea of 18-day-old fetal chick calvaria by treatment of the periostea with collagenase. The population was a mixture of different types of cells, comparable in its composition with the periosteum in situ, and included osteoblasts. After isolation, the cells were not cultured further but used immediately for short-term studies.

The periosteal fibroblast (PF) population was prepared by isolation of the cells from fresh periostea taken from both sides of fetal chick calvaria. Immediately after dissection the periostea were treated three times with collagenase (3 mg/ml, crude collagenase, Sigma), once for 10 min and then twice for 60 min. The first fraction (10 min), which was discarded, contained mainly erythrocytes and damaged cells. The two remaining fractions were combined, and the cells washed in Hanks' balanced salt solution (BSS) with 10% cock serum, resuspended in culture medium, and seeded in plastic Petri dishes. The culture medium consisted of M199 buffered with HEPES (20 mM, pH 7.4) and supplemented with 10% cock serum and 10% embryonic extract of 10-day-old chick embryos. After 3–4 days, the culture medium was replaced by fresh medium containing an amino acid–vitamin mixture instead of the embryonic extract (Table 1). Subsequently, the medium was changed every 3 days.

The third population, consisting of the osteoblasts (OB), was isolated from calvaria after removal of the periostea. Isolation and culture procedures were essentially the same as those used for the PF population.

After the calvaria were treated with collagenase for isolation of the OB population, the calvaria were washed thoroughly with 10% cock serum in Hanks and preincubated in M199 with 10% cock serum for 1 h. As a result of the removal of the majority of all other cell types, the cellular component of these collagenase-treated calvaria was very much enriched with osteocytes. These collagenase-treated calvaria were considered to be the fourth cell population (OC).

Scanning Electron Microscopy

Calvaria were fixed for 3 h at room temperature in 1.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and then post-fixed in 1% osmium tetroxide in the same buffer for 1 h. After fixation, the calvaria were held overnight in saline, dehydrated in graded acetone series, transferred to Freon TF, and critical point dried under Freon 13 in a Polaron critical point drying apparatus. They were then sputter-coated with gold in a Polaron Sputtercoater and examined with a Cambridge Stereoscan S180 at an acceleration voltage of 20 kV.

Table 1. Culture medium additions

Addition	mg/l M199
D-Glucose (BDH)	2000
L-Arginine (Merck)	100
L-Histidine, HCl (Sigma)	100
L-Lysine-HCl (Sigma)	150
L-Serine (Merck)	150
L-Proline (Sigma)	350
Glycine (BDH)	750
L-Cysteine HCl (Sigma)	90
L-Glutamine (Sigma)	100
L-Alanine (Sigma)	250
Niacinamide (Sigma)	20
Thiamine HCl (Sigma)	4
DL-α-Tocopherol acetate (Sigma)	1
Pyridoxine HCl (Sigma)	4
L-Ascorbic acid	200

Transmission Electron Microscopy

Calvaria were fixed in a cold solution of 0.66% osmium tetroxide and 0.83% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h, washed with buffer, and decalcified, if necessary, with 2.5% EDTA for 7 days in the cold. After post-fixation for 1 h in 1% osmium tetroxide in cacodylate buffer, the calvaria were immersed for 1 h in a solution of 0.25% uranyl acetate in acetate buffer (pH 6.3), dehydrated in alcohol, and embedded in Epon. Sections were strained with uranyl acetate and Reynolds' lead citrate and examined in a Phillips EM 200 [12].

In some experiments in which we wanted to avoid any possibility of decalcification, the calvaria were fixed in osmium tetroxide vapor for 1–3 h. After fixation, the calvaria were directly immersed in 100% alcohol and embedded in Epon. During sectioning, the sections were received in a trough filled with 0.035 M veronal acetate buffer (pH 8.4). They were examined either unstained or stained with lead citrate.

Incubations and Biochemical Determinations

For the determination of lactate and cAMP production and alkaline phosphatase activity, calvaria, OC samples (collagenase-treated calvaria), and PC suspensions were incubated in small incubation vessels. The PF and OB cultures were incubated in their culture dishes while still adhering to the bottom of the dish. The culture medium was generally Hanks' BSS, buffered with Hepes (20 mM, pH 7.4), containing 0.5% bovine serum albumin.

Lactate was determined according to Lowry et al. [13]. For the measurement of cAMP, a binding assay according to Lust et al. [14] was used after extraction of the cAMP from the tissue or cell cultures with *n*-propanol [15]. Protein and DNA contents of calvaria were determined in a 0.1 N NaOH extract after an extraction period of 16 h, protein according to Lowry et al. [16], DNA

according to Burton [17]. DNA contents of the cell cultures were determined according to Karsten and Wollenberger [18, 19], the protein content according to Lowry et al. [16] in a 0.1 N NaOH extract of the cultures. The alkaline phosphatase activity was determined by measuring the amount of NAD^+ which was formed from $NADP^+$ during 15–30 min of incubation of the calvaria or cell cultures [3].

Parathyroid hormone (bovine PTH, TCA powder, 260 U/mg protein) and prostaglandin E_1 were obtained from Sigma Chemical Company; calcitonin (salmon calcitonin, 2300 MRC units/mg) from Calbiochem; and Ro-20-2926 was kindly supplied by Dr. H. Sheppard of Hoffmann-LaRoche Inc. Purified bPTH (2500 U/mg, lot number 72/286) was a gift from the Medical Research Council.

Results

Histological Findings

When treated with collagenase after dissection, periosteal tissue disintegrated almost completely by the enzymatic digestion of the collagen matrix. Consequently, all cells present in the periostea were liberated by the collagenase treatment. Histological examination of the periostea after separation from the underlying bone showed that in addition to some blood cells and endothelial cells, they contained mainly fibroblasts. Osteoblasts were never found in these periostea; they remained on the surfaces of the calvaria or were destroyed during dissection. The PF population consists therefore mainly of fibroblasts. Although the periostea did not contain osteoblasts after dissection, for convenience we continued to call them periostea instead of outer layers of the periosteum, fibroblastic layer of the periosteum, or any other name.

When the periostea were cultured for 3 days on a solid coagulum, part of the cells in the cultures were still fibroblasts, but also other cell types had appeared: osteoblasts and cells with a larger and rounder nucleus than that of fibroblasts and with a less basophilic cytoplasm than is seen in osteoblasts (Fig. 1A, B). Judged from their position between the osteoblasts and the fibroblasts, they are probably osteoprogenitor cells or preosteoblasts. In the center of the cultures, between the double row of osteoblasts, an osteoid-like material was generally present (Fig. 1B). A more detailed description of these cultures has been given elsewhere [10]. From the histological findings we concluded that the PC population obtained from cultured periostea was composed of a mixed population of osteoblasts, fibroblasts, and preosteoblasts.

The origin of the cell population obtained by treating "bare" calvaria (without periostea) with collagenase was elucidated by studying the calvaria with SEM and TEM before and after treatment with collagenase. Before treatment, the surface as seen with SEM was covered with osteoblasts in some areas (Fig. 1C, D). Sometimes the osteoblasts formed an uninterrupted layer (Fig. 1C); sometimes the cells were separated from each other by gaps (Fig. 1D). In the latter case shrinkage during the critical point drying may have caused separation between the osteoblasts. In other areas the layer of osteoblasts had disappeared, probably as result of removal of the periosteum, exposing a surface of uncalcified collagen fibrils (Fig. 1E). In Fig. 1E lacunae can be seen among the collagen fibrils; some of these lacunae are empty, others contain osteocyte-like cells (Fig. 1F). The matrix underneath these cells was still uncalcified (Fig. 1G). We called these cells osteocytic osteoblasts because of their shape and position in lacunae on the one hand, and the absence of calcification of the surrounding matrix on the other hand. TEM confirmed the findings obtained by SEM (Fig. 2A, B). These osteocytic osteoblasts were also seen in calvaria fixed in osmium tetroxide vapor and processed further with minimal contact with aqueous solutions. In these calvaria artificial decalcification could be excluded. The organic matrix around these cells was uncalcified except for small isolated foci of crystallization (Fig. 2B).

During treatment of "bare" calvaria with collagenase, all noncalcified matrix was gradually dissolved. The heavily calcified matrix was not affected. After 1 h of enzymatic digestion the uncalcified collagen had already disappeared. In addition to osteocytes, at least some of the osteocytic osteoblasts were still present on the surface of the calvaria, to which they were attached by numerous cytoplasmic extrutions (Fig. 2C). After another hour of collagenase treatment, these cells too had disappeared from the surface (Fig. 2D). Osteocytes fully or half-embedded in the calcified matrix were not liberated by the collagenase, as could be concluded from the fact that we hardly ever found an empty lacuna on the surfaces of the calvaria after 2 h of treatment (Fig. 2D). Thus the OB population originated primarily from the layers of osteoblasts and osteocytic osteoblasts, whereas the OC population was a cell population heavily enriched with osteocytes.

Biochemical Observations

General. Under the conditions applied in this investigation, the numbers of cells in both the OB and the PF cultures increased about 10–20 times between the beginning of the third day and the end of the ninth day. The proliferation rate during the first

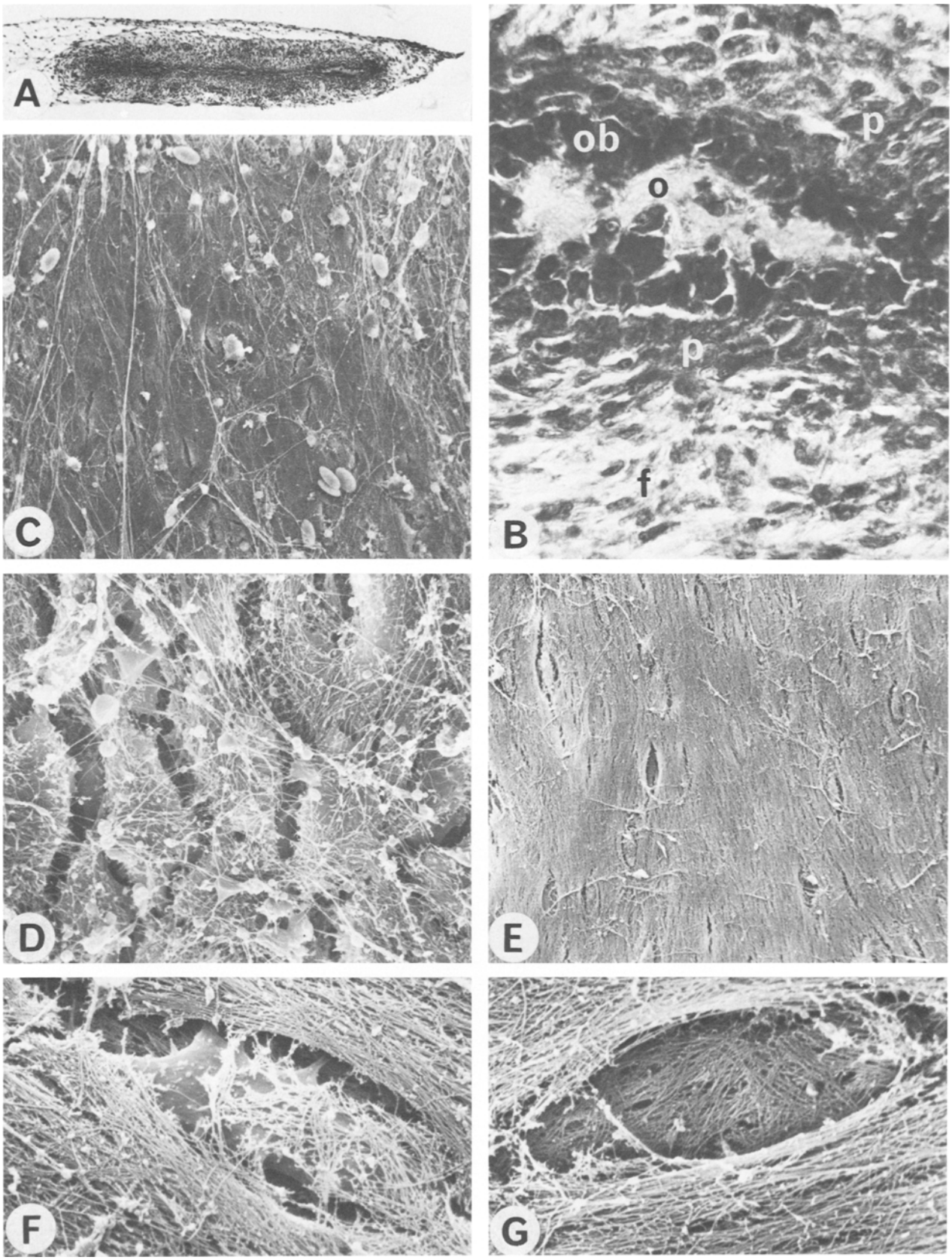

Fig. 1. **A** Azur-eosin-stained section of a periosteum, folded double after dissection and cultured for 3 days (×60). **B** Detail of a similar periosteum (×510). *ob*, osteoblast; *o*, osteoid; *p*, preosteoblast; *f*, fibroblast. **C–G** Scanning electron photomicrographs of the surface of a calvarium from which the periosteum was removed

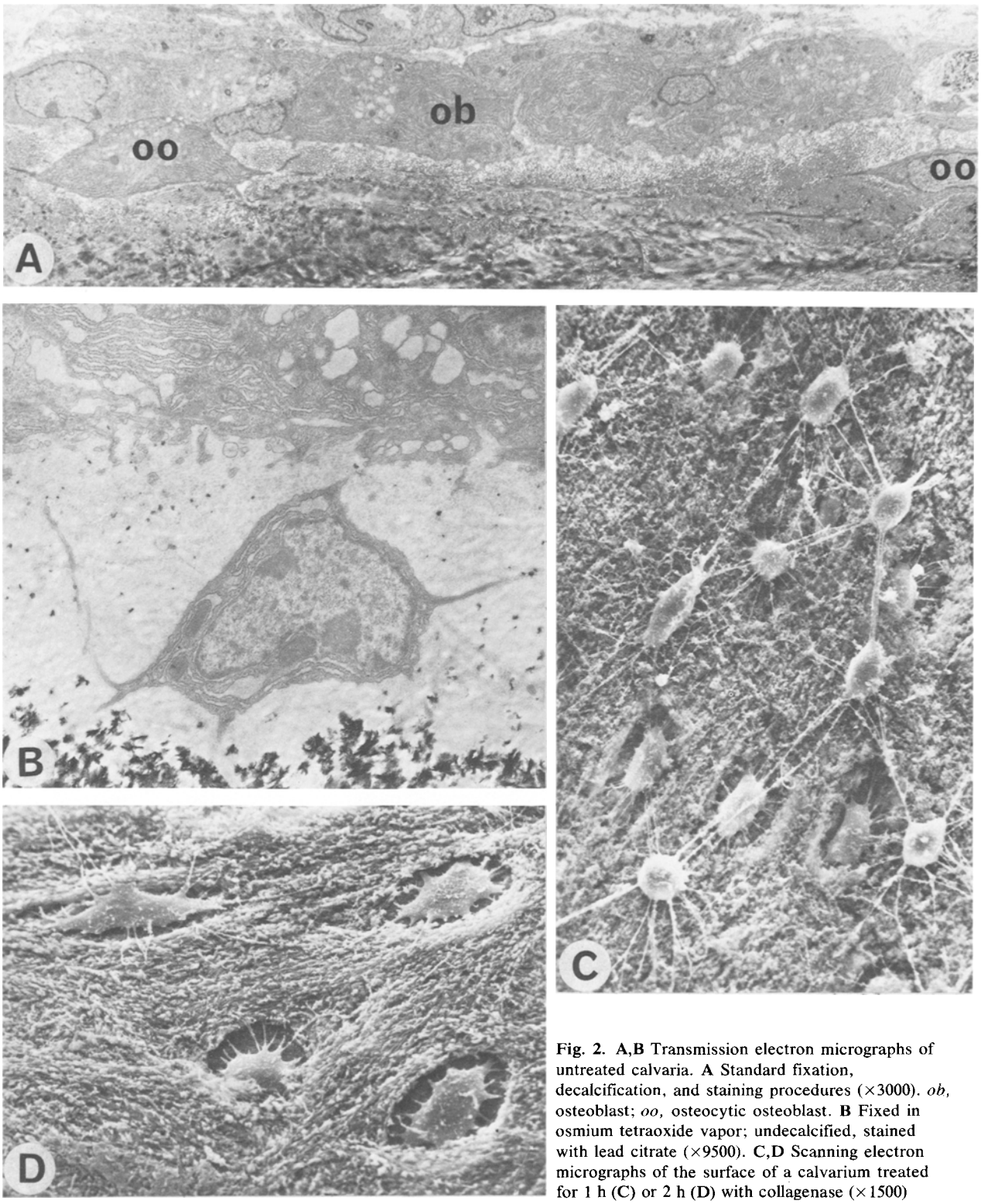

Fig. 2. **A,B** Transmission electron micrographs of untreated calvaria. **A** Standard fixation, decalcification, and staining procedures (×3000). *ob*, osteoblast; *oo*, osteocytic osteoblast. **B** Fixed in osmium tetraoxide vapor; undecalcified, stained with lead citrate (×9500). **C,D** Scanning electron micrographs of the surface of a calvarium treated for 1 h (C) or 2 h (D) with collagenase (×1500)

2 days was difficult to estimate. Only some of the isolated cells adhered to the bottom of the Petri dishes; the others remained floating in the medium. The latter, which were probably damaged cells not participating in proliferation, were usually removed during the first medium change. The cell proliferation of the OB and PF cultures is shown in Figs. 3 and 4, respectively, expressed as the amount of protein after increasing intervals of culture. In general, we have expressed the cellular activities of the

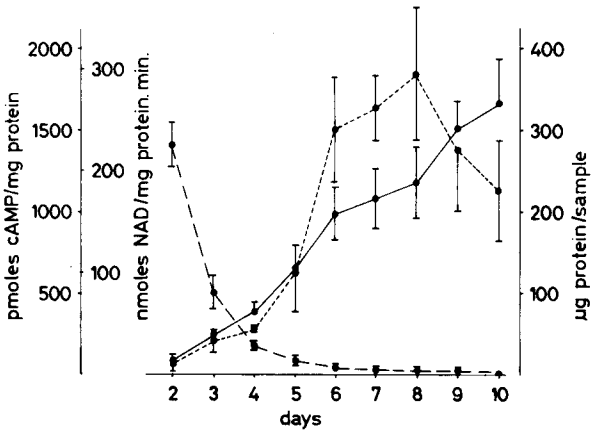

Fig. 3. Protein content, cAMP production, and alkaline phosphatase activity in relation to culture time in OB cell cultures. Means ± SE of 5 determinations. *Solid line*, protein content/sample; *dotted line*, cAMP production/mg protein; *broken line*, alkaline phosphatase activity/mg protein.min

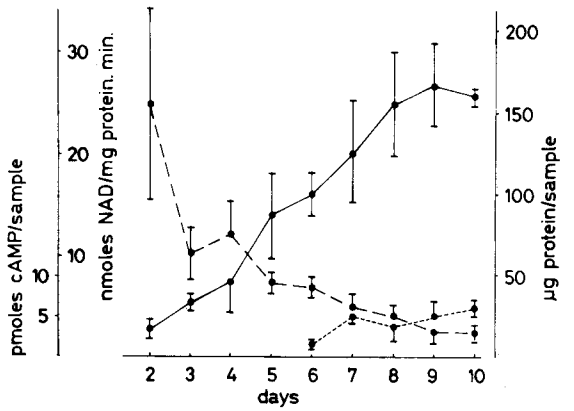

Fig. 4. Protein content, cAMP production, and alkaline phosphatase activity in relation to culture time in PF cell cultures. Means ± SE of 5 determinations. *Solid line*, protein content/sample; *dotted line*, cAMP production/sample; *broken line*, alkaline phosphatase activity/mg protein.min

different populations per amount of extractable protein, but it should be kept in mind when comparing the different types of cells that the DNA/protein ratio is not the same for all cell populations (Table 2). The OC population in particular has a much higher value for this ratio than does the PF or OB population. This may be due partially to the trypsin activity of the collagenase preparation with which the OC population was treated during the OB isolation procedure. Extracellular, extractable proteins are probably destroyed in the case of the OC population but will be isolated together with intracellular, extractable proteins in the case of intact calvaria and OB and PF cells.

In the OB population, alkaline phosphatase activity measured on the intact cultures started at a high level and remained on that level during the first 3 days of culture when expressed per sample, but decreased when expressed per milligram protein (Fig. 3). After 3 days the activity decreased further in both terms. The alkaline phosphatase activities of homogenates of OB cultures were higher and remained higher compared to the cell surface located activities of the cultures, but nevertheless decreased during culture (Table 3). The surface-located alkaline phosphatase activity of the PF population was much lower and also decreased during culture (Fig. 4).

In contrast with the high level of alkaline phosphatase activity, the sensitivity of the OB population to parathyroid hormone (PTH) with respect to cAMP production was quite low at the start of the culture period (Fig. 3). During the first few days the cells showed almost no response to PTH. After the fourth day the cAMP response, expressed as picomoles per milligram protein, increased considerably, reaching a maximum around day 8, and then decreased again (Fig. 3). The PF population virtually did not respond to PTH by increased cAMP production (Fig. 4). The very small response after 6–10 days may have originated from a small number of OB cells present in the PF preparations rather than from the PF cells themselves. The amount of cAMP in Fig. 4 is expressed as picomoles per sample instead of picomoles per milligram pro-

Table 2. DNA/protein ratio in various cell populations

Population	µg DNA/mg protein
Intact calvaria	40.7 ± 9.5 (50)
OC	115.3 ± 3.55 (24)
OB	43.1 ± 2.17 (9)
PF	44.5 ± 3.03 (6)

Values are given as means ± SE. Number of observations indicated in parentheses

Table 3. Alkaline phosphatase activities of intact and homogenized OB cultures

Culture time (days)	Alkaline phosphatase activity (nmoles NAD^+/µg DNA.min)	
	Homogenized cultures	Intact Cultures
2	2.55 ± 0.49	1.31 ± 0.27
4	0.88 ± 0.12	0.43 ± 0.09
7	0.78 ± 0.19	0.10 ± 0.02

All values are expressed as means ± SE of 10 observations. Both activities were measured on the same cultures

tein as in Fig. 3, becasue the small increase of the PTH-dependent cAMP production probably had nothing to do with the general increase in cell number. On the basis of these results, generally cultures of 7–9 days were used for the analysis of hormone responses in OB and PF cultures.

PTH-cAMP. The dose-response curve of the effect of PTH on cAMP production in the OB population is shown in Fig. 5, where the PTH concentration is expressed in moles per liter to facilitate comparison with Fig. 7. For calculating the concentrations in moles per liter we used a molecular weight of 10,000 for PTH and an activity of 2500 U/mg pure PTH. As Fig. 5 shows, PTH in high doses (2×10^{-8}–2×10^{-7}M) stimulated cAMP production enormously. At the relatively low dose of 2×10^{-10}M (inset of Fig. 5), PTH still caused a threefold increase of the cAMP content of the cells ($Pd < 0.05$, Student's t test for paired observations). In these studies 15 min was taken as the standard incubation time. Production of cAMP was maximal around that time (Fig. 6). Between 10 and 40 min after the introduction of PTH into the incubation medium, the cAMP response remained at this maximal level; after longer incubation periods the response decreased. In the presence of the phosphodiesterase inhibitor Ro 20-2926, the cAMP response to PTH stimulation was even more pronounced. When a maximal PTH dose was combined with a relatively high dose of Ro 20-2926 (2.5 mg/ml), exceptionally high cAMP levels were found in the cells (Table 4), although the inhibitor had no effect on the basal cAMP level (not shown). Furthermore, the peak of maximal stimulation shifted to longer incubation times. Nevertheless, after 2–4 h of incubation, the amount of cAMP in the cells had clearly decreased. When the media of the samples of group IV (see Table 4) were tested on fresh samples (group V), the response was generally lower than the response of group I, although not always as markedly as in the experiments shown in Table 4. The response was always higher than that of group IV. The decreased stimulatory activity of these reused media was not caused by a decrease in the amount of phosphodiesterase inhibitor. Ro 20-2926 preincubated for 4 h in the presence or absence of cell cultures enhanced the stimulatory activity of freshly added PTH to the same extent as nonpreincubated Ro 20-2926 (not shown).

Intact calvaria also showed an increased cAMP content after stimulation with PTH, although to a much smaller extent than the OB population. Removal of the periosteal and the osteoblastic layers (OC population) decreased the response

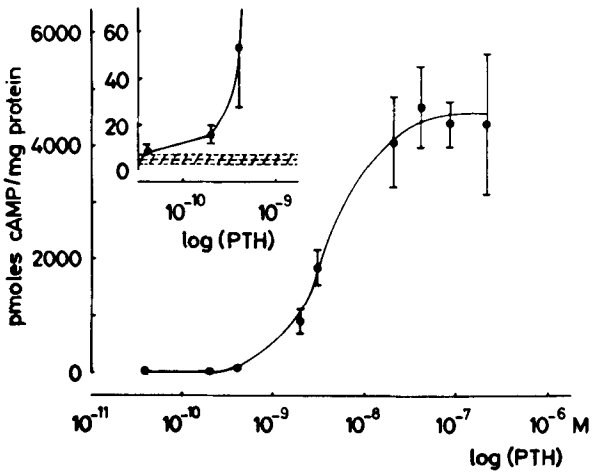

Fig. 5. PTH dose-response curve for OB cells. Means ± SE of 5 determinations

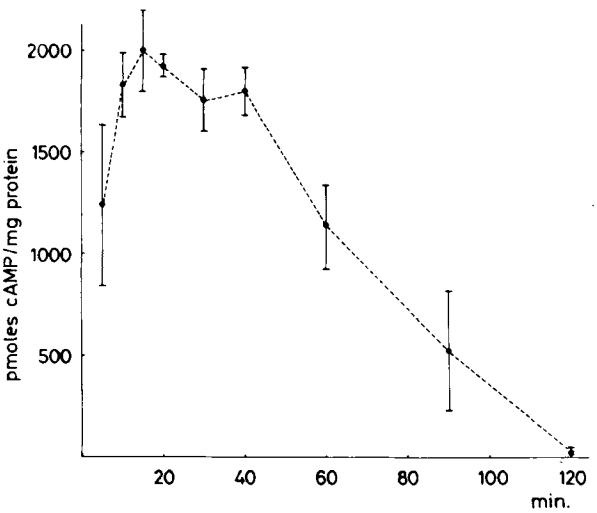

Fig. 6. Incubation time-response curve for PTH (1 U/ml) in OB cells. Means ± SE of 4 determinations

when expressed per calvarium but increased the response when expressed per milligram protein (Table 5).

The PC population did not show increased cAMP production after a PTH stimulus (1 U/ml), although osteoblasts were histologically demonstrable in the cultured periostea and although this population did show a PTH-stimulated Ca uptake [11].

Generally, a TCA-powder PTH preparation was used in the present experiments. To establish whether the observed effects of the hormone preparation were specific for PTH, the effects of purified bPTH-(1–84) were compared with those of the crude preparation in a concentration of 0.1 U/ml. Both preparations stimulated cAMP production in OB cells to about the same extent

Table 4. Effects of PTH (1 U/ml) in combination with Ro 20-2926 (2.5 mg/ml) on cAMP producton in OB cells

Group	Incubation time (min)	cAMP in pmoles/mg protein		P
		Mean	SE	
I	15	10,040	610	I–II < 0.001
II	60	18,440	1,550	
III	120	12,270	1,360	III–II < 0.02
IV	240	5,260	440	IV–V < 0.01
V (Medium of group IV)	15	7,590	420	V–I < 0.02

All values are expressed as means ± SE of 10 observations. Statistical evaluation according Student's *t* test for unpaired observations

Table 5. Effects of PTH (1 U/ml) on cAMP production in calvaria

	cAMP		N	P
	pmoles/calvarium	pmoles/mg protein		
Control calvaria	5.4 ± 0.34	14.4 ± 1.0	17	
Calvaria + PTH	28.2 ± 2.26	66.7 ± 5.6	17	< 0.001
OC population + PTH	13.8 ± 1.74	342.5 ± 37.1	20	< 0.001

Values are given as means ± SE. Incubation time 15 min. Significance of the difference between either of the PTH-treated groups and the control group tested with the Student's *t* test for unpaired observations

Table 6. Effects of PTH (1 U/ml) on lactate production

Population	Lactate in μmoles/mg protein.h		Stimulation (%)	P
	Control	PTH		
Intact calvaria	0.105 ± 0.013 (18)	0.132 ± 0.012 (18)	26	< 0.02[a]
PC	0.102 ± 0.009 (10)	0.138 ± 0.013 (10)	35	< 0.01[a]
PF	0.285 ± 0.046 (6)	0.324 ± 0.054 (6)	14	< 0.02[a]
OB	0.296 ± 0.008 (19)	0.390 ± 0.012 (19)	32	< 0.001[b]
OC	0.82 ± 0.046 (8)	1.24 ± 0.120 (8)	51	< 0.01[b]

Values are given as means ± SE. Number of observations in parentheses
[a] Student's *t* test for paired observations
[b] Student's *t*-test for unpaired observations

(means ± SE, TCA-PTH: 643 ± 111, purified PTH: 428 ± 76 pmoles/mg protein); there was no significant difference ($N = 15$, Student's *t* test for unpaired observations).

PTH-Lactate. PTH stimulated lactate production in all cell types and in intact calvaria (Table 6). Lactate production rates in PF, OC, and OB populations were about equal when expressed per mg DNA (compare with Table 2). However, in view of the comparatively low lactate production rate in intact calvaria and cultured periostea, the relatively high production rates of PF and OB populations are perhaps not representative of the production rates in vivo. Culture conditions may have been responsible for this increased level of glycolysis.

Again, to establish the specificity of the observed effects, the increased lactate production rates of OB, PF, and OC populations in the presence of TCA-powder PTH preparation were compared with the production rates in the presence of an equal amount of purified bPTH-(1-84). Both preparations, TCA-PTH and purified PTH, stimulated lactate production significantly ($P < 0.01$, $N = 5-9$) with, respectively, 62.3% and 47.7% for OB, 54.5% and 28.6% for PF, and 35.3% and 29.0% for OC; there were no significant differences between the stimulatory effects of the two PTH preparations.

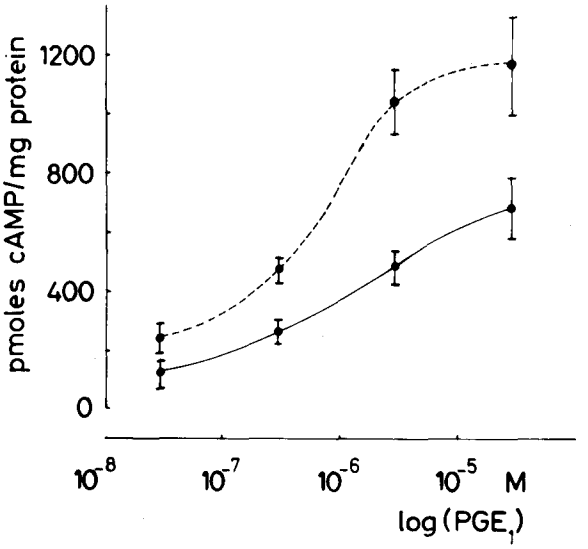

Fig. 7. PGE_1 dose-response curve in OB (*solid line*) and PF (*dotted line*) cells. Means ± SE of 8 (OB) or 6 (PF) determinations

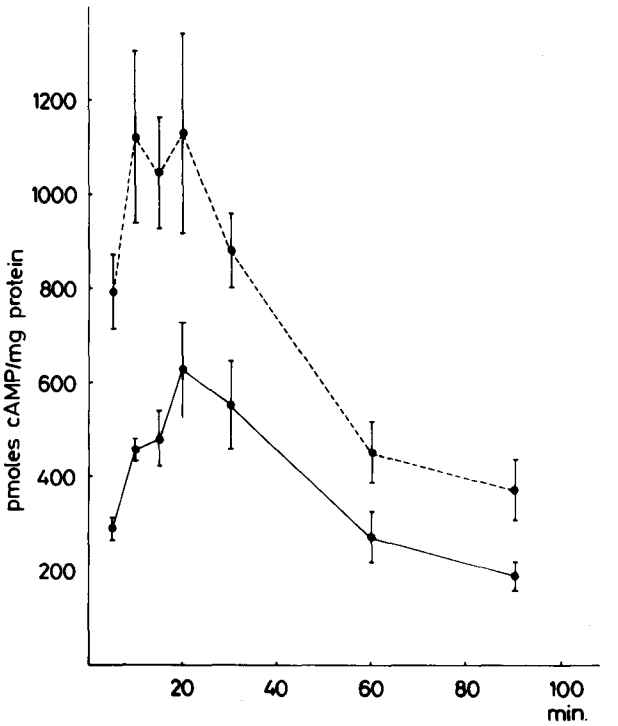

Fig. 8. Incubation time-response curve for PGE_1 (3×10^{-6}M) in OB (*solid line*) and PF (*dotted line*) cells. Means ± SE of 5 (OB) or 4 (PF) determinations

Prostaglandin E_1-cAMP. Like PTH, prostaglandin E_1 stimulated cAMP production in the OB population, but unlike PTH it also stimulated cAMP production, and even more, in the PF population (Fig. 7). The time course of the stimulation, in OB as well as in PF, was similar to that of the PTH stimulation of the cAMP production in OB cells (Figs. 6 and 8). However, as shown in Fig. 7, the level of maximal stimulation was much lower and the concentration PGE_1 needed for maximal stimulation much higher compared with PTH.

Calcitonin. We found no effect of calcitonin on the cAMP production in the OB, PF, or PC populations in the concentration range of 1 to 100 mU CT per ml. When tested in the same concentration range, CT also had no effect on the PTH (0.1 U/ml) induced cAMP production stimulation in OB cells (not shown).

Discussion

Isolation and culture of bone cells has been described by others [1, 2, 6–8]. For the present study, however, we used calvaria of fetal chicks instead of those from fetal mice or rats. Fetal chicks have the advantage of being cheaper and easier to handle. Moreover, the periosteum is much easier to remove from a fetal chick calvarium than from a fetal mouse or a fetal rat calvarium, which means less damage to the underlying layer of osteoblasts.

The surfaces of the calvaria we studied with SEM and TEM were at least partially covered with osteoblasts and closely resembled the descriptions given by other investigators [20, 21]. The osteoblasts seemed to form a unicellular layer of closely apposed cells, which effectively separated the bone matrix from the outer side of the periosteum. Underneath this layer we found a layer of noncalcified matrix containing cells situated relatively far apart. These cells were morphologically similar to osteocytes, although generally larger. Their position between the layer of osteoblasts and the calcified matrix suggests that these cells probably play an important role in the calcification of the bone matrix. We have called these cells provisionally osteocytic osteoblasts. The osteocytic osteoblasts appeared to be connected with osteoblasts, osteocytes, and each other by numerous cytoplasmic extrusions. We have never found the same type of cell in radii of fetal mice [22, 23]. In radii, calcification of the bone matrix starts quite close to the osteoblastic layer. Osteocytic osteoblasts may therefore be characteristic for calcification in calvaria or, perhaps more likely, for calcification in the chick.

Histological study of the calvaria before and after treatment with collagenase showed that it is possible to separate osteoblasts from periosteal fibroblasts and from osteocytes. The biochemical re-

sults are in agreement with this, especially with respect to the differences in alkaline phosphatase activity and the differences in PTH-stimulation of cAMP production between the OB and PF populations. Morphologically, cultured OB cells are difficult to distinguish from cultured PF cells. In culture, both cell types appear to be fibroblast-like cells. Macroscopically, cultures of the two cell types differ somewhat in that OB cells are able to grow in multicellular layers, whereas PF cultures tend to be mono- or bilayers.

Although the high cell surface located alkaline phosphatase activity of the OB cells immediately after isolation and the marked PTH stimulation of cAMP production after 6–9 days of culture both define the OB cell as an osteoblast-like cell [8], the culture time-dependence curves of the two parameters (Fig. 3) seem difficult to reconcile with each other. Measurement of the alkaline phosphatase activity in homogenates of cell cultures showed that the total amount of activity also decreased during culturing but to a much smaller extent than the surface-located activity alone (Table 3). The difference between the two activities (total minus surface) represents probably intracellular phosphatase activity although inaccessibility for the substrate of a large proportion of the cells in multilayer cultures during the surface-located activity determination cannot be excluded. We have not yet found a way to avoid loss of activity in the cultures. High-density cell seeding together with the use of a bone fluid-like medium may offer a remedy [24].

The cAMP curve in Fig. 3 can be explained in three ways: (a) isolated osteoblasts dedifferentiate into progenitor cells [21] that are relatively insensitive to PTH; the progenitor cells proliferate and after 3–4 days differentiate again into osteoblasts; (b) a mixed population of osteoblasts and fibroblasts is isolated from the "bare" calvaria; the former cells are very much in the minority but during culture proliferate much faster than the fibroblasts; and (c) the collagenase treatment has damaged the PTH receptors. The second possibility is unlikely in view of the histological observations. The third possibility is also not very likely. The time between the isolation and the reappearance of PTH sensitivity (3–4 days) is too long for such an explanation because repair mechanisms would certainly have been activated during such a period. Moreover, formation of new undamaged cells has taken place during this period.

The decrease in the stimulation of cAMP production after 9–10 days (Fig. 3) is probably caused by deterioration of the cell cultures. Confluency is usually reached after 8–10 days. When the cells are kept in culture even longer, the cell layer becomes detached from the bottom of the dish and curls up, and the cells perish. The presence of embryonic extract (EE) in the culture medium promoted this process, which is why a medium without EE is substituted after 3–4 days, even though EE is an excellent stimulator of cell proliferation.

The time course of the cAMP response to PTH administration in OB cultures (Fig. 6) is similar to that found for fetal rat calvaria [25], but the decline in the amount of cAMP occurs later and is also less steep. This decline is probably the result of a number of processes. Desensitization (M. P. M. Herrmann-Erlee, *personal communication*) and/or intracellular phosphodiesterase activation might explain the lower level of cAMP in group IV (Table 4) compared with group V. The difference between groups I and V can be explained only by cleavage [26] and/or adsorption of parathyroid hormone to the incubation vessel [27] or by the release of a PTH antagonist during the 4 h of incubation [28]. The same processes that govern the cAMP level after PTH stimulation are probably responsible for the shape of the cAMP time curve after PGE_1 administration.

All cell types investigated in this study were stimulated by PTH as far as lactate production is concerned. Moreover, in earlier studies [11, 29] we showed that calcium uptake in the PC population can be stimulated by relatively low concentrations of PTH (0.01 U/ml). In other words, all cell types studied in this investigation have PTH receptors. Since PTH stimulates cAMP production in OB and OC cells and PGE_1 stimulates cAMP production in OB and PF cells, all cell types must have adenylate cyclase in the cell membranes. These findings combined with the fact that PC cells do not show any stimulation of cAMP production after administration of PTH, although cultured periostea contain cells morphologically identified as osteoblasts (Fig. 1A,B) and although one expects osteoblasts to show cAMP stimulation [30], led us to formulate the following hypothesis: all cell types (OC, OB, PF, and PC) have PTH receptors and adenylate cyclase activity, but the interaction between receptor and enzyme is not possible in periosteal fibroblasts, preosteoblasts, and young osteoblasts. This hypothesis also offers an explanation for the lack of stimulation of cAMP production in the OB population during the first days of culture (Fig. 3). A similar phenomenon of differentiation of hormone-insensitive cells (as far as adenylate cyclase is concerned) to hormone-sensitive cells during cell culture has been described for adipocytes by Rubin et al. [31].

Finally, the problem remains of the lack of effect

of calcitonin of cAMP production in OB, PF, and PC populations. On the basis of the work done by Cohn et al. [6–8] and Peck et al. [32], one would have expected calcitonin to stimulate cAMP production in PF and PC cells. We have found no calcitonin-mediated stimulation in cultures of PF cells but also not in periostea or periosteal cells immediately after isolation [32]. However, the difference in sensitivity of the cells isolated from the periosteal regions of calvaria of fetal or neonatal mouse, rat, and chick may be a reflection of the difference in the number of osteoclasts present in the calvaria of these species. When calvaria of near-term mice, rats, and chicks are stained histochemically for succinic dehydrogenase activity [33], which is a marker for osteoclasts [34], mouse calvaria are found to contain far more osteoclasts than do rat calvaria, whereas chick calvaria contain almost none (Nijweide, unpublished results).

Acknowledgments. The authors are indebted to the Laboratory for Electron Microscopy of the University of Leiden, especially to Mr. H. K. Koerten. The skillful technical assistance of Miss J. K. Danes is also gratefully acknowledged. This work was supported by the J. A. Cohen Institute for Radiopathology and Radiation Protection, Leiden.

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