ON THE MECHANISMS OF BONE RESORPTION

The Action of Parathyroid Hormone on the Excretion and Synthesis of Lysosomal Enzymes and on the Extracellular Release of Acid by Bone Cells

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ABSTRACT

Bone resorption, characterized by the solubilization of both the mineral and the organic components of the osseous matrix, was obtained in tissue culture under the action of parathyroid hormone (PTH). It was accompanied by the excretion of six lysosomal acid hydrolases, which was in good correlation with the progress of the resorption evaluated by the release of phosphate, calcium 45 or hydroxyproline from the explants; there was no increased excretion of two nonlysosomal enzymes, alkaline phosphatase, and catalase. Balance studies and experiments with inhibitors of protein synthesis indicated that the intracellular stores of the acid hydrolases excreted were maintained by new synthesis. The release was not due to a direct disruption of the lysosomal membrane by PTH; it is presumed to result from an exocytosis of the whole lysosomal content and to involve mechanisms similar to those controlling the secretion of this content into digestive vacuoles.

The resorbing explants acidified their culture fluids at a faster rate and released more lactate and citrate than the controls; this release was in good correlation, in the PTH-treated cultures, with the resorption of the bone mineral, but the amount of citrate released was considerably smaller than that of lactate. The acid released could account for the resorption of the mineral.

It is proposed, as a working hypothesis, that the acid hydrolases of the lysosomes are active in the resorption of the organic matrix of bone and that acid, originating possibly from the stimulation of glycolysis, cares for the concomitant solubilization of bone mineral while also favoring the hydrolytic action of the lysosomal enzymes.

INTRODUCTION

A concomitant removal of the organic and of the mineral components of the extracellular matrix of bone is a general character of bone resorption, regardless of the type of the operant cell: osteoclast (Kölliker, 1873; Hancox, 1956), osteocyte (Bélanger et al., 1963) or, possibly, other cells (Cameron et al., 1967).

However, the chemical identity of the agents responsible for these phenomena and the nature of the cellular mechanisms involved in their excretion is still a matter of speculation. The participation of enzymes able to hydrolyse collagen, proteins, and mucopolysaccharides has been postulated (McLean and Urist, 1961) and

supported by observations of collagenolytic factors in bone (Walker et al., 1964; Woods and Nichols, 1965). Similarly, the hypothesis of a secretion of chelating agents (McLean, 1954) or of acid (Weidenreich, 1930) has been put forward to explain the removal of the mineral and it received credit from studies on the metabolism of bone cells (for a review, see Vaes, 1966 *b*).

It is the purpose of the present paper to report and to discuss observations on biochemical and cytological processes which are presumed to be of importance in the digestion of the organic matrix: namely the synthesis and the excretion of several lysosomal hydrolases by the cells while resorbing the extracellular framework of bone. Data which may have significance regarding the mechanisms of solubilization of the mineral (the release of acid and of large amounts of lactate by the cells) will also be reported, and, in the discussion, an hypothesis will be presented which integrates these two groups of observations. Parts of the work described have already been the object of preliminary notes (Vaes, 1965 *b*, 1966 *a* and *b*).

MATERIALS AND METHODS

To obtain a model system of bone resorption, advantage was taken, throughout this study, of the property of PTH[1] to stimulate this process, either in tissue culture or in vivo.

Bone Resorption in Tissue Culture

Calvaria (without the occipitals) were taken off 18–19 days Swiss albino or NMRI mouse embryos, put in a drop of Hanks' solution and dissected aseptically under a Zeiss stereomicroscope; their weights varied from 1.5 to 3.5 mg, according to the stage of development. They were then introduced into 20 ml—plastic roller-tubes (1 calvarium per tube, as a rule) and laid into 0.05 ml chicken plasma, at 2 cm from the bottom of the tube. Fixation of the explants on the wall by clotting of the plasma was favored by leaving the tubes in the horizontal position during ½ hr at room temperature before the addition of the culture fluid; unless otherwise specified, this consisted in 1 ml of medium 199 containing Hanks' salt solution as a base (Morgan, et al., 1955). The tubes were laid at 37°C in an almost horizontal position on the roller-tubes drum (6 revolutions per hr) and cultivated aseptically under an atmosphere of air. The culture media were renewed after 1 or 2 days of culture (exceptionally, 3 days). Due to the low buffering capacity of medium 199, when equilibrated with air, its pH shifted from 7.4 to 8.3–8.5 during the first hours of culture at 37° by loss of CO_2; the acidification of the culture fluids by the explants was thus manifested by a progressive lowering of the pH below 8.3–8.5.

The calvaria were treated in a paired manner. Two calvaria were taken from embryos of the same litter and cut at equal sizes under the dissecting microscope; 1 USP unit/ml of PTE was added to the medium of the "resorbing" calvarium and appropriate amount (0.01 ml) of a substitute solution (0.2% phenol, 1.6% glycerol and 0.7% cristalline bovine serum albumin), to the "control." Critical experiments were repeated, using purified PTH (2500 USP units per mg) or the nonhormonal parathyroid peptides 2, 3S, and 5 (Hawker et al. 1966) instead of the impure whole-gland extract used routinely; the solvent (0.001 N acetic acid) was then added alone to the controls. As the calvaria contain, besides bone, some fibro-cartilaginous tissue present at the sutures in between the individual bones constituting them, control experiments were also done in which carefully dissected parietals, containing only bone tissue, were put into culture; the two parietals from the same embryo were then paired.

For the experiments requiring labeling of the embryonic bones with radioisotopic calcium 45, the pregnant mice were injected subcutaneously, 64 hr (unless otherwise specified) before being killed, with 40–90 μc of ^{45}Ca labeled calcium chloride (specific activity: more than 1 c/g Ca).

Bone Resorption In Vivo

Infant Wistar rats of either sex and up to 8 days of age (generally, between 4 and 6 days) were injected subcutaneously twice daily, at 9 a.m. and at 5 p.m., during 3 days, with either 10 USP units of PTE or an equal volume (0.1 ml) of the control solution already described; Control and PTE rats were paired within the same litter. They were killed by decapitation 15–16 hr after the last injection; the serum inorganic phosphorus concentrations were then markedly depressed in the PTE group ($-33 \pm 4.6\%$; $p < 0.001$). Their whole calvaria were dissected out, immersed in ice-cold 0.25 M-sucrose and used as material for the preparation of homogenates or of cytoplasmic fractions.

[1] Abbreviations used: C, controls; EDTA, ethylenediamine tetraacetate; N, number of determinations; N.S., nonsignificant; p, level of significance; PTE, parathyroid extract; PTH, parathyroid hormone; SD, standard deviation.

Preparation and Fractionation of Homogenates of Bone Tissue

Pooled calvaria were minced with scissors and homogenized in 2–3 ml of 0.25 M-sucrose with a motor-driven, ground-glass conical homogenizer (Potter and Elvehjem, 1936); homogenates which had not to be further fractionated were made directly in bidistilled water.

The homogenates were centrifuged at 600 *g* during 10 min at 0°C, yielding a cell-free supernatant ("cytoplasmic extract" or E fraction) and a sediment containing most of the nuclei together with cell debris, connective tissue elements and solid mineral (N fraction). In some experiments, the cytoplasmic extract was further fractionated by differential centrifugation, according to Vaes and Jacques (1965 *a* and *b*) to yield M, L, P, and S ("nonsedimentable") fractions. Nonsedimentable enzyme activities are expressed in per cent of the total activities assayed in the reconstituted homogenates (sum of fractions E and N).

Cultures of Isolated Fibroblasts

Fibroblasts, obtained by trypsinization from the whole carcass of eviscerated 19-day rat embryos, were grown as monolayers (Paul, 1961) during 1 wk in a modified Eagle (1959) minimal essential medium supplemented with 10% calf serum; the main modifications included the substitution of 0.5% lactalbumin hydrolysate to the amino acid present in Eagle's recipe as well as increasing the glucose concentration from 0.1 to 0.45%. Subcultures (Paul, 1961), containing about 4×10^5 cells in 3 ml medium, were cultivated in Leighton tubes with appropriate refeedings, during either 1–3 days or 7 days, to obtain, respectively, actively growing or almost stationary cultures. They were then further cultivated during 1 or 2 days in fresh medium (either Eagle-serum or medium 199) containing variable amounts of PTE or of its substitute solution. This last medium was used for the enzymatic and chemical analyses.

Enzyme Assays and Analytical Procedures

Most of the techniques used for the enzyme assays have been described in detail elsewhere (Vaes and Jacques, 1965*a*). The definition of the "free activities" of the acid hydrolases and the methods used for their measurement have also been reported (Vaes, 1965*a*). Units of enzyme activities generally refer to the decomposition of 1 μmole of substrate/min, except for cytochrome oxidase, catalase, and hyaluronidase, whose units have been described together with their assays (Vaes and Jacques, 1965*a*).

In some experiments, acid phosphatase was determined by the amount of *p*-nitrophenol liberated from *p*-nitrophenyl phosphate. The reaction was carried out in 0.5 ml of 0.008 M-*p*-nitrophenylphosphate and 0.1 M-acetate buffer, pH 5.0, and stopped by addition of 2.5 ml of 0.1 N-NaOH containing 0.01 M-EDTA. The absorbancy was read at 405 mμ, after filtration. Under these conditions, the extinction coefficient of a *p*-nitrophenol standard was 1.6×10^4 $cm^{-1}M^{-1}$. The rate of reaction was proportional to enzyme concentration up to, at least, 2.5 mg of calvaria (cytoplasmic extract) and constant with time up to 30 min.

When the enzyme assays were done on the tissue culture media, these were first centrifuged at 600 *g* during 10 min, immediately after their removal from the roller-tubes, to discard any floating cells. The blanks for the enzyme assays included then media "cultivated" without calvarium. Due to their low activities in the culture media, *N*-acetyl-β-glucosaminidase, β-galactosidase and cathepsin required 18–24 hr of incubation and acid *p*-nitrophenylphosphatase, 2 hr. Although the latter reactions were then no longer linear with time, they were still directly proportional to the enzyme concentrations, both in the absence and in the presence of PTE: thus the absolute total amounts of these enzymes released in the culture fluids were underestimated by these measurements, but valid quantitative comparisons between experimental groups were still allowed. The other enzymes measured in the culture fluids and all the enzymes measured in the tissues were assayed in conditions where the reactions were directly proportional both with enzyme concentrations and incubation time.

Considerable analytical difficulties were encountered in the measurement of cathepsin in the tissue culture media, due to high and variable blanks; this measurement could not be done reliably on the first culture medium. To obtain more precise data for the activities released on the second day of culture (see Table I), Hanks' solution was substituted for medium 199 after the 1st day of culture; medium 199 was however used to measure the activity released on later days.

DNA was determined by the fluorimetric method of Kissane and Robbins (1958), lactate, by the enzymatic method of Horn and Bruns (1956), citrate, by the method of Natelson et al. (1948), phosphate by the method of Marinetti et al. (1959). Hydroxyproline was determined by the method of Bergman and Loxley (1963), either directly, to measure the free amino acid, or after hydrolysis in sealed tubes in 6 N—HCl for 3 hr at 138° (culture media) or for 22 hr at 110° (bone tissue), to measure the total hydroxyproline (free + peptide-bound). Hexosamine was measured by the method of Cessi and Piliego (1960) after hydrolysis of the tissue in 5N-HCl for 4 hr at 100° (Dingle et al. 1966).

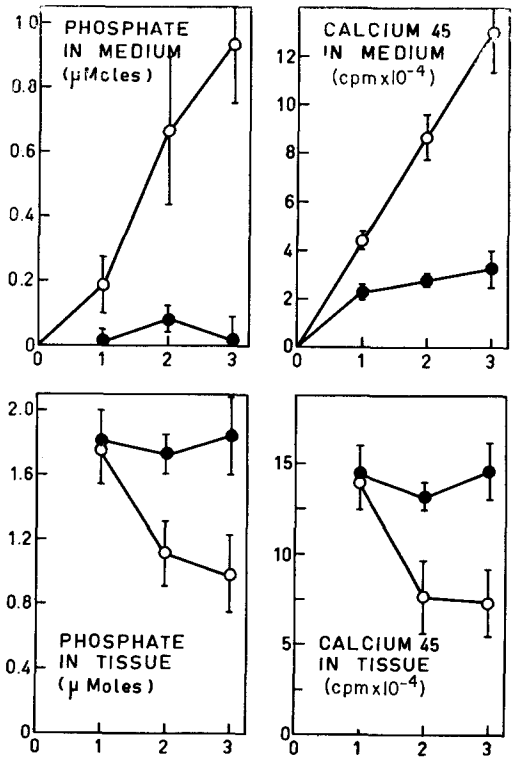

FIGURE 1 Resorption of the mineral from ^{45}Ca-labeled calvaria explants under the action of PTE. The explants were cultivated during 1, 2, or 3 days in 2 ml of medium; the phosphate and the ^{45}Ca content of the culture fluids and of the tissues were then measured. Each point is the mean of 5 cultures; vertical bars represent ±1 S.D. PTE, open symbols; Controls, full symbols.

Radioactive calcium was measured in a liquid scintillation counter for both the samples of culture medium and the acid extracts of the bone mineral (1 mouse embryo calvarium for 1 ml 1 N-HCl).

Statistics

Statistics in the tables and figures refer to means ± SD. The significance, p, of the differences observed between groups (usually paired) were determined with the use of Student's *t* test.

Materials

Purified PTH was a gift from Dr. Howard Rasmussen (The University of Pennsylvania School of Medicine, Philadelphia, Pa.) and from Dr. Gerald D. Aurbach (National Institutes of Health, Bethesda, Md.); Dr. Rasmussen also provided several non-hormonal parathyroid peptides. Subcultures of fibroblasts in Leighton tubes were donated by Dr. André Trouet, from this laboratory. PTE (Para-Thor-Mone) was obtained from Eli Lilly & Co., Indianapolis, Ind., medium 199 (without phenol red) and desiccated chicken plasma, from Difco Laboratories, Detroit, Mich., and *p*-Nitrophenyl-phosphate, from Sigma Chemical Co., St. Louis, Mo. The source of substrates used for the other enzyme assays is given in Vaes and Jacques (1965*a*).

RESULTS

Bone Resorption in Tissue Culture

Resorption lacunae appeared in the parietals on the first day of culture with PTE or PTH and extended rapidly in the whole calvaria on the following 2 days, forming actual holes in the tissue (see Vaes, 1965 *b* and 1966 *b*). Histological examinations showed the presence of numerous osteoclasts, which could also be visualized, following the technique of Barnicot (1947), by their ability to concentrate rapidly (within 45 min) the neutral red added to the cultures at the final concentration of 1/10000 (w/v); these cells were then seen mostly at the edges of the resorption lacunae.

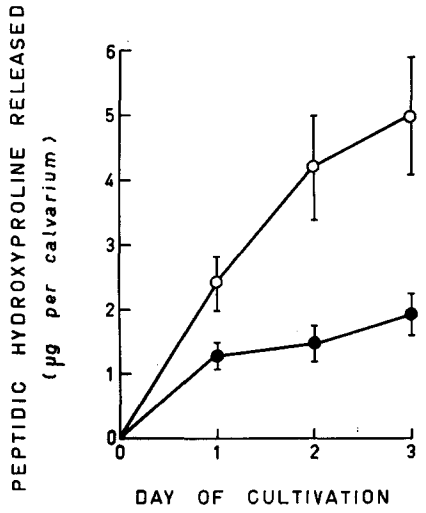

FIGURE 2 Effect of PTE on the release of peptide-bound hydroxyproline by calvaria explants into their culture fluids. The explants (3 calvaria per tube) were cultivated during 3 days in 1.6 ml of medium; the culture fluids were renewed every day. The graphs represent the cumulative release of peptide-bound hydroxyproline. Each point is the mean of 4 culture tubes; vertical bars represent ±1 S.D. PTE, open symbols; Controls, full symbols.

TABLE I

Release of Enzymes into Culture Medium by Calvarium Explants Undergoing Active Resorption under the Influence of PTE

The enzymes were assayed in the media removed from the culture-tubes at various time intervals and corresponding to different periods of culture (day 1, day 2, day 1 + 2, day 3,etc.). The number of pairs of cultures, C and PTE, assayed in each subgroup is given between brackets. The differences observed between C and PTE are significant at $p < 0.001$ for all subgroups, except for cathepsin at day 2 and 3 + 4 ($p < 0.005$); for β-galactosidase, at day 4 ($p < 0.01$); for alkaline phosphatase, at day 1 ($p < 0.05$) and at day 2 (N.S.); for catalase, at day 1 and 2 + 3 (N.S.).

		Enzyme recovered from the culture fluid (micro-units per calvarium)							
Day of culture		β-glucuronidase	β-galactosidase	Acetyl-β-glucosaminidase	Acid deoxyribonuclease	Cathepsin	Acid paranitrophenyl phosphatase	Alkaline phosphatase	Catalase
1	C	(32) 36 ± 7	(11) 46 ± 12	(11) 380 ± 43			(6) 419 ± 86	(15) 5827 ± 1914	(9) 73 ± 17
	PTE	63 ± 18	71 ± 15	925 ± 148			798 ± 92	6501 ± 2148	67 ± 17
2	C	(9) 16 ± 4	(13) 13 ± 4	(9) 274 ± 63		(40) 62 ± 32	(6) 141 ± 19	(14) 1616 ± 338	
	PTE	46 ± 4	36 ± 13	623 ± 87		104 ± 72	582 ± 67	1583 ± 524	
1 + 2	C		(23) 57 ± 19		(12) 352 ± 75			(11) 8144 ± 760	
	PTE		115 ± 40		420 ± 85			5584 ± 1311	
2 + 3	C	(12) 28 ± 4							(4) 28 ± 10
	PTE	84 ± 21							17 ± 9
3	C	(9) 18 ± 3	(10) 6 ± 1	(9) 336 ± 54			(6) 96 ± 32	(17) 1613 ± 1034	
	PTE	72 ± 8	27 ± 6	982 ± 181			502 ± 77	684 ± 446	
4	C	(14) 25 ± 7	(6) 8 ± 1	(9) 370 ± 58					
	PTE	99 ± 26	22 ± 6	1139 ± 252					
3 + 4	C		(10) 24 ± 9		(12) 97 ± 24	(21) 134 ± 59			(10) 44 ± 7
	PTE		69 ± 29		314 ± 104	187 ± 84			24 ± 7
4 + 5 + 6	C	(12) 104 ± 22							
	PTE	293 ± 59							
5 + 6 + 7	C		(23) 36 ± 18	(10) 742 ± 136	(8) 52 ± 20	(18) 280 ± 87			
	PTE		80 ± 30	2847 ± 497	518 ± 175	430 ± 142			

Over the first 3 days of culture, the resorbing ^{45}Ca-labeled calvaria had lost about half of their inorganic phosphate and of their ^{45}Ca content (Fig. 1). The control calvaria did not lose inorganic phosphate; their small release of ^{45}Ca probably reflects an exchange of isotopic calcium with ^{40}Ca from the culture fluid. When, for each day of culture, the phosphate and the ^{45}Ca released in the medium were expressed as percentage of the corresponding element in the whole culture system (medium + tissue), the differences between PTE and controls were found to be closely similar, on each day, for both elements (respectively for phosphate and ^{45}Ca, 10.2 and 11.5% on day 1; 33 and 35.6% on day 2, 48 and 47.7% on day 3), indicating a homogeneous labeling of the bone mineral by the isotopic calcium. There were however some differences in the amounts of phosphate or of ^{45}Ca recovered, on the various days, in the total culture system (medium + tissue), due to the variability between the masses of the individual bones put into culture.

Over the first 2 days of culture, the resorbing calvaria had lost 28.7% ($p < 0.005$) of their hydroxyproline (initial content: 14.6 ± 2.5 μg per calvarium; N = 8) and 23.3% ($p < 0.01$) of their hexosamine (initial content: 4.3 ± 0.6 μg per calvarium; N = 8) contents; these losses were respectively of 6.9% (N.S.) and 7% (N.S.) in the controls. Peptide-bound hydroxyproline (but not free hydroxyproline) was also recovered in greater quantities from the media of the resorbing explants (Fig. 2).

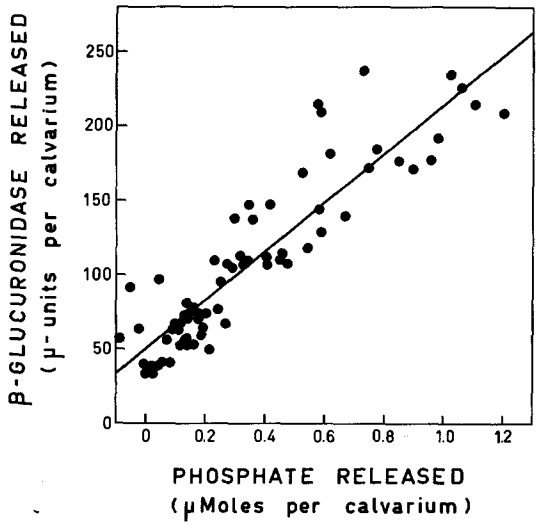

FIGURE 3 Correlation between the excretion of β-glucuronidase and the progress of bone resorption evaluated by the amount of inorganic phosphate released from the calvaria under the action of PTE ($r = 0.905$). The explants were cultivated during 1, 2, or 3 days either without or with increasing concentrations (0.01, 0.05, 0.1, 0.5, or 1 unit per ml) of PTE, in order to obtain a wide range in the extension of the resorption processes.

There were no significant differences between the DNA of the bone cultivated for 3 days in the presence or in the absence of PTE (respectively 11.0 ± 2.0 and 11.1 ± 3.1 μg per calvarium; N = 8).

Excretion of Acid Hydrolases

During the development of bone resorption, increasing amounts of acid hydrolases were released by the cells into their culture fluids as compared to the nonresorbing controls: this was observed for β-glucuronidase, *N*-acetyl-β-glucosaminidase, β-galactosidase, acid deoxyribonuclease, acid *p*-nitrophenylphosphatase and cathepsin. In contrast, the extracellular release of other enzymes, such as alkaline phosphatase or catalase, was not modified at the beginning of the resorption and it was even decreased after a few days (Table I). More enzyme was always recovered from the first culture medium (C or PTE) than from the following media, presumably due to leakage of enzymes from the cells which were wounded by the explantation processes.

β-glucuronidase, and sometimes also acetyl-β-glucosaminidase, were used as reference acid hydrolases for further studies of this phenomenon. Their release was also increased when pure PTH was substituted for PTE at the same dose level (1 USP unit, corresponding to 0.4 μg PTH, per ml) or when isolated parietal bones only, instead of whole calvaria, were cultivated with PTE.

The increased release of β-glucuronidase was not observed when the tissue was cultivated with PTE but under conditions which did not allow the development of resorption, for instance, after treatment with actinomycin D or with puromycin (see next section). PTE (0.1–2 units/ml) was also without effect on the cellular release of β-glucuronidase and of *N*-acetyl-β-glucosaminidase, when it was added to cell cultures of rat fibroblasts. On the other hand, a good correlation was observed between the excretion of β-glucuronidase and the progress of resorption in the tissue evaluated either

morphologically, by a semiquantitative estimation of the extension of the resorption lacunae under the dissecting microscope, or by a quantitative measurement of the inorganic phosphate (Fig. 3 and Table II) or of the hydroxyproline (Fig. 4) released from the bones into the culture media when the explants were cultivated for various lengths of time with increasing concentrations of PTE. Time-course studies showed also that the solubilization of the mineral, as determined by the release of ^{45}Ca from labeled bone, and the excretion of reference acid hydrolases by the cells, appeared almost simultaneously in this system, both being first detected as early as in between the 1st and the 4th hr after the beginning of the culture in some experiments (Table III).

The possibility that an increase in the concentration of calcium in the fluids bathing the cells was the cause of the release of the acid hydrolases could not be substantiated; increasing that concentration in the culture fluid up to a saturation point did not modify the release of β-glucuronidase by the explants. On the other hand, a decrease in that concentration or the addition of EDTA to the culture fluid increased the release of β-glucuronidase and of acetyl-β-glucosaminidase by the cells (Table IV).

Synthesis of Acid Hydrolases

A balance study was made by considering the hydrolases which were present in the tissue on the second and on the 7th day of culture and those which were excreted in between these 2 days (Table V). On day 2, the resorbing calvaria contained generally a little less acid hydrolases than the controls but on day 7, the opposite tendency was observed: they contained equal or higher quantities of acid hydrolases than the controls, although they had excreted 3–4 times more of these enzymes into the culture fluid. The behavior of alkaline phenylphosphatase was quite different: the resorbing bones lost this enzyme at a faster rate than the controls (Table V) although, as already seen in Table I, they released less of it into the culture fluid. In another group of experiments, increased activities were also found for other acid hydrolases (cathepsin, acid β-glycerophosphatase, acid phenylphosphatase, acid *p*-nitrophenylphosphatase) in calvaria cultivated for 3 days in the presence of PTE (Table VI).

Calvaria cultivated in the presence of PTE, did not resorb if they were pretreated with actinomycin D or if they were treated simultaneously with puromycin. In these conditions, the effect of PTE to increase the excretion of β-glucuronidase was either abolished (Fig. 5) or greatly diminished (Fig. 6). Moreover, at the end of the culture period, the β-glucuronidase content of the calvaria treated with the inhibitor was only 35–40% of the content of the nontreated explants and the differences observed in this content between C and PTE explants were no longer significant.

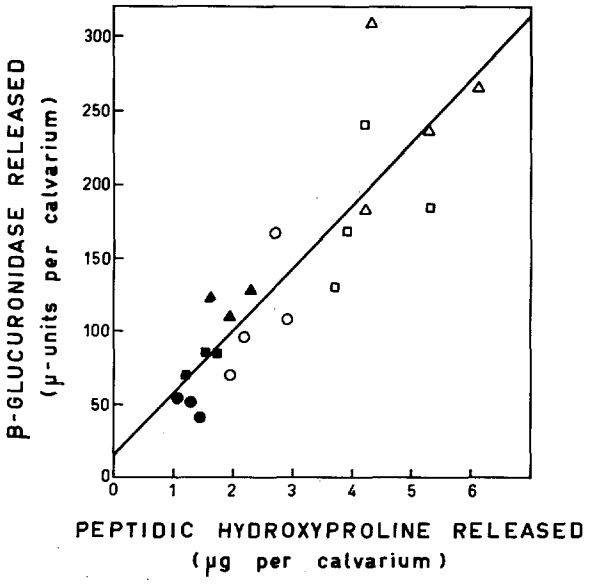

FIGURE 4 Correlation between the excretion of β-glucuronidase and the progress of bone resorption evaluated by the amount of peptide-bound hydroxyproline released from the calvaria (r = 0.872). The explants were cultivated during 1 (circles), 2 (squares) or 3 (triangles) days either without (full symbols) or with PTE (open symbols).

Experiments with Calvaria from Infant Rats

The activities of several acid hydrolases were increased in homogenates of calvaria of young rats which had been treated in vivo by PTE, while that of the mitochondrial cytochrome oxidase, the reference enzyme, was unchanged (Table VII, column A). Moreover, an increased proportion of the acid hydrolases was found either as "nonsedimentable" enzymes in the homogenates (Table VII, column B), or as "free" enzymes in the cytoplasmic extracts prepared from these homogenates (Table VII, column C).

However, PTE had no significant effect on the latency of β-glucuronidase when it was added directly to cytoplasmic extracts or to combined M + L fractions of bone cells in vitro (Table VIII).

Excretion of Acid, of Lactate, and of Citrate

The rate of acid production by the explants was stimulated by the presence of PTE. This effect was already noticeable after 8 hr culture and also when isolated parietals were cultivated instead of the whole calvaria (Fig. 7). It was obtained with purified PTH but not with three nonhormonal peptides (2, 3S, and 5) isolated from parathyroid gland extracts by Rasmussen et al. (1964) and by Hawker et al. (1966) (Table IX).

PTE, as well as pure PTH, stimulated considerably the release of lactate by the resorbing bones into their culture medium (Table X); it

TABLE II

Effect of the Concentration of PTE in the Culture Fluid on the Release of β-Glucuronidase, of Inorganic Phosphate, and of Lactate by Explanted Calvaria

β-glucuronidase activity is expressed in micro-units, and phosphate and lactate, in micromoles released per calvarium over 3 days. Each subgroup contained four cultures.

Units PTE per ml	Amount released over 3 days		
	β-glucuronidase	Phosphate	Lactate
0	101 ± 16	0.24 ± 0.19	4.0 ± 0.4
0.01	122 ± 40	0.29 ± 0.27	4.5 ± 0.5
0.05	193 ± 36	0.79 ± 0.25	5.2 ± 0.8
0.10	175 ± 26	0.66 ± 0.20	5.1 ± 1.0
0.50	207 ± 21	0.93 ± 0.25	5.9 ± 0.4
1.00	207 ± 24	0.89 ± 0.18	6.8 ± 0.7

TABLE III

Early Effects of PTE on the Resorption of the Mineral (mobilization of ^{45}Ca) and on the Release of Acid Hydrolases by Calvaria Explants in Culture

The media were renewed at the end of each period of cultivation. ^{45}Ca was injected to the pregnant mice 64 hr before their sacrifice in experiments A but only 24 hr before in experiments B. The results are expressed in per cent of the quantities measured for paired controls, considered as 100%.

Experiment and period of cultivation	N	Relative activity $\left(\frac{PTE}{C} \times 100\right)$			
		^{45}Ca	Acetyl-β-glucosaminidase	β-glucuronidase	Lactate
A.					
0–2 hr	6	104 ± 15	103 ± 34	100 ± 25	140 ± 79
0–4 hr	12	104 ± 13	109 ± 25	99 ± 19	135 ± 42*
0–6 hr	13	119 ± 16‡	123 ± 32§	107 ± 18	128 ± 16§
4–8 hr	8	126 ± 23*	192 ± 57*	142 ± 38‡	183 ± 70‡
6–10 hr	8	162 ± 35§	297 ± 13§	216 ± 87§	208 ± 126*
B.					
0–1 hr	8	107 ± 14	97 ± 18	96 ± 16	—
1–4 hr	8	120 ± 17	127 ± 32*	128 ± 42	—
4–7 hr	8	138 ± 23§	257 ± 86§	740 ± 532§	—

* $p < 0.05$.
‡ $p < 0.01$.
§ $p < 0.005$.

TABLE IV

Influence of Calcium and of EDTA on the Release of β-Glucuronidase and Acetyl β-Glucosaminidase by Calvaria Explants into their Culture Fluids

The culture media used are described in the table; calcium (Ca) was added as calcium chloride and EDTA, as a solution adjusted to pH 7.4. Each subgroup contained four to six cultures. The results reported were obtained on the 2nd day of culture for group 1 and between the 30th and 47th hr of culture for groups 2 and 3; for these last two groups, the explants had been first cultivated during 30 hr in medium 199, with or without PTE. Addition of EDTA directly to the enzyme assays, at appropriate concentrations, had no effects on the activities of these enzymes.

Culture medium	Micro-units released per calvarium	
	β-glucuronidase	Acetyl-β-glucosaminidase
1. Medium 199		
a) 1.3 mM Ca (normal)	49 ± 18	—
b) 2.3 mM Ca	48 ± 15	—
c) 3.3 mM Ca	52 ± 14	—
d) 6.3 mM Ca (saturation)	47 ± 18	—
2. Hanks medium		
A. without PTE		
a) 1.3 mM Ca (normal)	19 ± 3	102 ± 22
b) Ca-free Hanks	22 ± 8	151 ± 44*
c) Ca-free Hanks + EDTA (2 mM)	33 ± 4‡	253 ± 62‡
B. with PTE		
a) 1.3 mM Ca (normal)	45 ± 10	266 ± 104
b) Ca-free Hanks	50 ± 7	355 ± 101
c) Ca-free Hanks + EDTA (2 mM)	63 ± 6*	560 ± 35‡
3. Medium 199		
a) 1.3 mM Ca (normal)	33 ± 22	81 ± 65
b) + EDTA (3 mM)	51 ± 25*	127 ± 76

* $p < 0.05$.
‡ $p < 0.005$.

had no effect on the lactate production of fibroblasts in culture when it was used at concentrations ranging from 0.1 to 2 units/ml. Simultaneously, it increased the release of citrate by the resorbing bones but the excess of citrate released over the controls under PTE was, on a molar basis, about 30 times smaller than the excess of lactate excreted over the controls under the same influence.

Good correlations were observed, but only in the PTE- or PTH-treated cultures, between the release of ^{45}Ca from the mineral and that of lactate (Fig. 8) and of citrate (Fig. 9) by the cells; no such correlations were observed in the control cultures. Similarly, good correlations were found, in the PTE- or PTH-treated cultures, between the release of inorganic phosphate from the mineral and that of lactate (Fig. 10) or of citrate ($r = 0.969$) but they were not observed in the control cultures, neither for lactate (Fig. 10) nor for citrate ($r = -0.297$).

Time-course studies (Table III) showed that the increased release of lactate by PTE occurred very soon after the beginning of the cultures, accompanying or even preceding the first signs of bone mineral solubilization, as determined by the release of ^{45}Ca from labeled bone. It was proportional to the concentration of PTE present in the culture fluid (Table II). As discussed in the next section, it could be correlated with the increased release of acid and with the solubilization of phosphate from the bone mineral that occurred under PTH-stimulation (Table XI).

TABLE V

Balance of Enzyme Activities between the 2nd and 7th day of Cultivation for Control and Resorbing Calvaria

Enzyme activities were measured in homogenates prepared from pooled explants after either 2 or 7 days (6 days for β-glucuronidase) cultivation: the number of pairs (C and PTE) of homogenates assayed is given between brackets. The enzyme activities are compared to those recovered from the medium between day 2 and 7, as calculated from Table I.

Enzyme		Micro-Units Per Calvarium			
		In tissue on day 2	Recovered from medium between day 2 and 7	In tissue on day 7	Excess over day 2
β-glucuronidase	C	(8) 340 ± 46	147	(6) 390 ± 91	197
	PTE	300 ± 26‡	464	380 ± 36	544
Acetyl-β-glucosaminidase	C	(8) 3400 ± 560	1448	(6) 3600 ± 910	1648
	PTE	3000 ± 320*	4968	4800 ± 580‡	6768
β-galactosidase	C	(8) 620 ± 37	60	(6) 520 ± 65	−40
	PTE	600 ± 23	149	630 ± 11‡	179
Alkaline phosphatase	C	(8) 81000 ± 9600	—	(7) 60000 ± 7800	—
	PTE	62000 ± 8300§	—	38000 ± 12000‡	—

* $p < 0.05$.
‡ $p < 0.01$.
§ $p < 0.005$.

TABLE VI

Effect of PTE on the Cathepsin and Acid Phosphatases Content of Explanted Calvaria

Enzyme activities were measured in six to nine homogenates prepared from pooled explants cultivated for 3 days with or without PTE.

Enzyme	Micro-units per calvarium	
	C	PTE
Cathepsin	1236 ± 138	1400 ± 120*
Acid β-glycerophosphatase	1804 ± 438	2387 ± 343*
Acid *p*-nitrophenylphosphatase	7176 ± 2007	21040 ± 3217‡
Acid phenylphosphatase	3210 ± 1967	12701 ± 5703‡

* $p < 0.02$.
‡ $p < 0.005$.

DISCUSSION

Bone Resorption in Tissue Culture

Gaillard (1959 and 1961) first demonstrated the possibility of stimulating bone resorption by PTE in tissue culture. His original culture system differs from ours by the use of complex natural media and of stationary cultures, but the evolution of the resorption process appears similar in both systems, although somewhat faster in ours. He followed, through microcinematography, the development of resorption in the matrix of parietals from mouse embryos and noticed that the phenomenon occurred in contact or in close vicinity of osteoclasts; he suggested that these cells could solubilize the matrix through the secretion of dissolving agents.

As seen in the present paper, biochemical consequences of this resorption are, as expected (see Martin et al., 1965), the release of calcium and phosphate from the bone mineral into the culture fluid. Simultaneously, hydroxyproline,

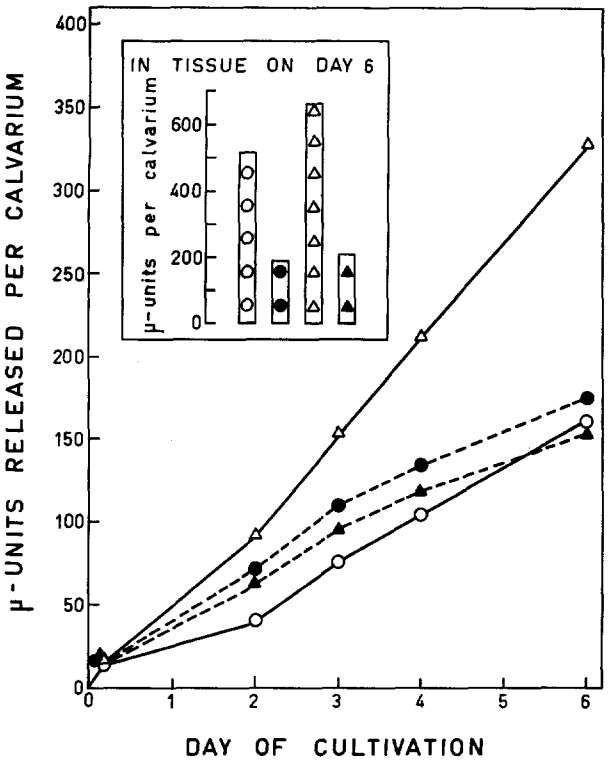

FIGURE 5 Effects of actinomycin D on the excretion and on the synthesis of β-glucuronidase by calvaria in culture. The explants were precultivated during 5 hr with (full symbols) or without (open symbols) actinomycin D (0.1 μg per ml), washed with Hanks' solution and further cultivated with (triangles) or without (circles) PTE. Bone resorption did not develop in the PTE-explants after pretreatment with this concentration of actinomycin D (0.05 μg per ml still allowed development of resorption lacunae in two explants over four while 0.01 μg per ml had no effect). The main graph reports the cumulative excretion of β-glucuronidase over 6 days cultivation and the upper graph, the quantities of β-glucuronidase present in the pooled explants after the 6th day of culture. Each point is the mean of 5–6 cultures.

an inbuilt collagen marker (Udenfriend, 1966), is liberated from the fibers of the matrix and recovered, mostly in peptide form, in the culture medium. Moreover, hexosamines are lost by the explants, presumably from their mucopolysaccharides; the nature of the hexosamine-containing fragments liberated in the culture fluid was not determined in the present study. Thus not only the mineral, but also the normally insoluble organic constituents of the matrix are simultaneously solubilized.

Exocytosis of Lysosomal Enzymes and Resorption of the Organic Matrix

Of particular interest was the observation that the development of bone resorption was paralleled by the release of six acid hydrolases in the culture medium.

The enzyme activities, cited throughout this paper as released by the explants, were in fact those which were recovered from the culture fluids after variable lengths of cultivation. The actual quantities of enzymatic proteins released could however be greater than those found in the culture medium if enzyme inactivation occurs in the medium. When culture fluids, from either control or resorbing calvaria, were further cultivated during 24 hr in the absence of tissue or cells, almost no loss of activity was found for some enzymes (β-glucuronidase, alkaline phosphatase) while a variable loss was encountered for others (−20–30% for cathepsin and deoxyribonuclease, −40–60% for acid *p*-nitrophenylphosphatase and acetyl-β-glucosaminidase, −50–90% for β-galactosidase). Thus, some of the figures presented for the quantities of enzymes released may have to be increased by a factor which varies depending on the enzyme considered and on the conditions of the culture. The extention of enzyme inactivation, although somewhat variable, was generally similar in the media from the PTE or from the control cultures. Thus, the differences observed between the enzyme activities measured in the culture fluids from resorbing and from control bones cannot be explained on the basis of selective inactivation.

Previous studies on bone enzymes (Vaes and Jacques, 1965 *a* and *b*; Vaes, 1965 *a* and 1967) have shown that the hydrolases released in the culture media are normally associated with the

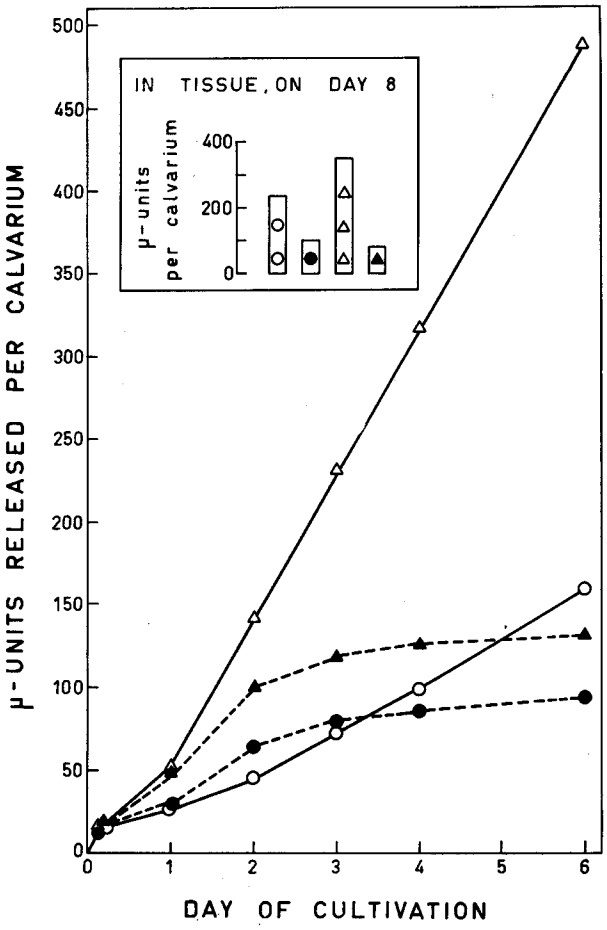

FIGURE 6 Effects of puromycin on the excretion and synthesis of β-glucuronidase by calvaria in culture. The explants were cultivated during 6 days with (triangles) or without (circles) PTE and in the presence (full symbols) or in the absence (open symbols) of puromycin (1 μg per ml). Development of resorption in the PTE-treated calvaria is exceptional when the tissues are cultivated with this concentration of puromycin; it did not occur in the present experiments. The main graph represents the cumulative excretion of β-glucuronidase over 6 days cultivation and the upper graph, obtained in a separate experiment, the quantities of β-glucuronidase present in the explants after the 8th day of culture. Each point is the mean of 5–6 cultures.

lysosomes of this tissue, together with other enzymes, hyaluronidase, acid ribonuclease, acid β-glycerophosphatase and acid phenylphosphatase (acid *p*-nitrophenylphosphatase was not considered in these studies, but is likely, from preliminary experiments, to be closely similar to acid phenylphosphatase). The lysosomal enzymes were simultaneously released from the particles present in the homogenates of bone under the action of various agents, noxious to the lysosomal membrane (Vaes, 1965 *a*). The present experiments, showing the simultaneous release of six lysosomal hydrolases by actively resorbing bone cells, suggest strongly that the whole enzymatic content of the lysosomal "bag" may also be excreted in bulk from the cells under the action of PTH, presumably by the process of exocytosis (de Duve, 1963; de Duve and Wattiaux, 1966). That such a phenomenon also occurs in vivo, is suggested by the increased proportion of soluble or of free acid hydrolase activities present in the homogenates of bones taken from rats treated with PTE, a fact which may be interpreted (although other interpretations are possible: see de Duve, 1963 and 1965) as reflecting the presence of more soluble lysosomal enzymes in the extracellular spaces of the tissue after stimulation of their exocytosis by the hormone.

The only other mechanism which could explain the simultaneous release of lysosomal enzymes, is cell necrosis. However, this possibility seems very unlikely in our system, for the following reasons. First, the release of acid hydrolases occurred at the very beginning of the culture period and coincided with the initiation of bone resorption. Second, enzymes, such as alkaline phosphatase or catalase, which are not associated with the lysosomes (Vaes and Jacques, 1965 *b*; Vaes, 1965 *a*), were not released in greater amounts by the resorbing bones than by the controls. Third, there was no difference in the DNA content of the

TABLE VII

Effect of PTE-Treatment on the Total, the Nonsedimentable and the Free Activities of Acid Hydrolases in Calvaria from Infant Rats

The results are expressed in per cent of the activities measured for paired controls, considered as 100%. Each column of the table refers to an independent group of experiments. Nonsedimentable activities are the means of the proteins or of the enzymes assayed in the S fraction after three paired fractionations of whole homogenates of calvaria. Free and total activities are the means of six or seven paired determinations made either on cytoplasmic extracts (free activities or hyaluronidase total activity) or on whole homogenates (all the other total activities) of calvaria.
For an evaluation of the significance of nonsedimentable and of free activities, see de Duve (1964 and 1965).

Enzyme	Relative activity $\left(\frac{PTE}{C} \times 100\right)$		
	A. Total	B. Nonsedimentable	C. Free
β-glucuronidase	124 ± 17*	164 ± 13§	119 ± 11‡
Acetyl-β-glucosaminidase	111 ± 16	—	135 ± 29‡
β-galactosidase	110 ± 6‡	158 ± 10§	109 ± 8*
Hyaluronidase	114 ± 13*	—	—
Cathepsin	126 ± 10‡	121 ± 22	95 ± 5
Acid deoxyribonuclease	138 ± 24*	189 ± 71	114 ± 7‡
Acid phenylphosphatase	146 ± 22‡	—	110 ± 16
Acid β-glycerophosphatase	—	—	108 ± 12
Cytochrome oxidase	99 ± 11	—	—
Proteins	—	147 ± 20*	—

* $p < 0.05$.
‡ $p < 0.01$.
§ $p < 0.005$.

bones cultivated for 3 days in the presence or in the absence of PTE; since there is no morphological evidence for a more rapid rate of *renewal* of osteoclasts or other bone cells under continuous PTH-stimulation (for a review, see Vaes 1966 *b*) and, more specifically, in PTE-treated explants closely similar to ours (Gaillard, 1959), this lack of change in the DNA content suggests that no significant loss (or gain) of cells occurred under PTE. Fourth, no pictures of evident necrosis were seen on histological preparations of the resorbing bones after their cultures. Fifth, if the release were due to necrosis, it should be accompanied by a depletion of the stores of the acid hydrolases in the tissue; in fact, these stores were not depleted, but increased.

The release of lysosomal enzymes could be a consequence of bone resorption. Osteoclasts resorbing bone have an intense pinocytotic activity which is located at their ruffled border, immediately under the extracellular zone where the first signs of resorption are noticed (for a review, see Vaes, 1966 *b* and 1969). In the light of what is known about the physiology of the lysosomes (de Duve and Wattiaux, 1966), it is conceivable that the intracellular digestion of the matrix fragments taken in this manner leads to the formation of residual bodies containing indigestible material together with active lysosomal enzymes. Bulk discharge of the contents of these bodies, through a sort of "cellular defecation" (de Duve and Wattiaux, 1966) could occur at the nonresorbing pole of the osteoclast and then be the cause of the simultaneous release of several lysosomal enzymes outside of the resorption zone. However, the fact that the release of enzymes was already demonstrated after 1–4 hr cultivation with PTE, at a time when the resorption of the mineral was only at its very beginning, seems to exclude this possibility.

It appears more probable that the release of lysosomal enzymes, which is clearly correlated with bone resorption, is not a consequence, but a cause of this resorption. Although this is only an hypothesis, it fits not only the present data on PTH-stimulated bone resorption but also, as

TABLE VIII

Influence of PTE on the Latency of β-Glucuronidase in Cytoplasmic Extracts of Calvaria from Infant Rats

The cytoplasmic extracts were incubated at 37° in a medium buffered at pH 5, during 30, 60, or 120 min, in the presence of 1 or 10 units of PTE per ml or of an equivalent amount of the PTE substitute solution. The results of group 4 were obtained in a separate experiment, with a combined M + L fraction preincubated during 30 min at pH 7.5 (1 unit PTE per ml) or at pH 6.8 (10 units PTE per ml). The free activity is expressed in per cent of the total activity measured in the same extract or fraction in the presence of 0.1% Triton X-100; the total activity is not influenced by the presence of the hormone or of its substitute.

Experimental group	Free activity (% of total)			
	1 unit/ml		10 units/ml	
	C	PTE	C	PTE
1. 30 min at pH 5	48.7	49.1	48.7	51.0
2. 60 min at pH 5	49.4	52.1	51.1	52.7
3. 120 min at pH 5	51.9	54.0	52.1	57.5
4. 30 min at pH 7	45.2	46.2	46.9	43.7

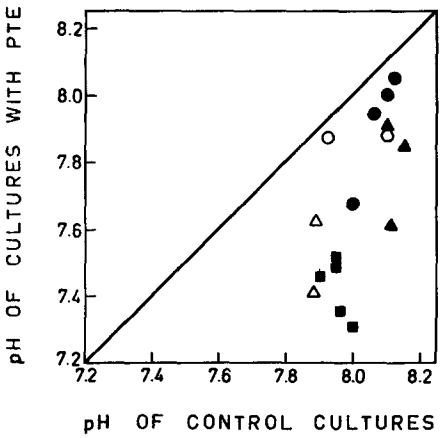

FIGURE 7 Acidification of the culture fluids of resorbing embryonic bones. Various amounts of tissue were cultivated during various lengths of time and the pH of the culture fluid was then immediately measured with a glass electrode; each point refers to two paired cultures, with or without PTE. The differences of pH observed between PTE and controls could not be accounted for by the slightly smaller acid buffering capacity of the medium containing PTE as compared to the control medium containing the substitute solution. Full circles, two calvaria cultivated during 8 hr; open circles, one calvarium cultivated during 24 hr; full triangles, one calvarium cultivated during 46 hr; open triangles, one calvarium cultivated during 71 hr; squares, four parietal bones cultivated during 72 hr.

reviewed more extensively elsewhere (Vaes, 1969), what is presently known of the ultrastructural, cytochemical, and cytological aspects of bone resorption. Ultrastructural studies show that the first stages of the osteoclastic bone resorption are extracellular: the organic fibers of the matrix are first dissociated (Robinson, 1965), apparently through digestion of interfibrillar, mucopolysaccharidic, ground substance (Scherft, 1968), and this is followed by the loss of the typical cross-striation of the collagen fibers (Hancox and Boothroyd, 1963; Scherft, 1968), presumably under the action of collagenolytic proteases. However, these digestions will proceed with some efficiency only if the mineral embedding these elements is either previously or simultaneously removed (Neuman et al., 1960).

This requires that the osteoclasts excrete in the resorption zone underlying them agents which dissolve the mineral and depolymerize the insoluble constituents of the matrix. Several histochemical studies (Burstone, 1960; Handelman et al., 1964; Doty and Robinson, 1968) have shown the presence of acid phosphatase at this level, and this has been confirmed under the electron microscope (Doty and Robinson, 1968). These studies may be accepted as a morphological counterpart of the excretion of at least one lysosomal enzyme in the resorption zone underlying the osteoclast. Considered together with the evidence presented

TABLE IX

Effect of Purified PTH and of Three Nonhormonal Parathyroid Peptides on the Rate of Acid Production by Calvaria in Culture

The calvaria were cultivated during 48 hr in 1 ml medium 199 containing one of the following supplements: PTE, PTH, peptide 2, peptide 3S or peptide 5 (Hawker et al., 1966), the PTE substitute solution or 0.001 N acetic acid, the solvent of the parathyroid peptides.

The pH of the culture fluids were determined at the end of the culture period. Each subgroup contained three or four cultures.

Experiment and group	Additive to the culture medium	pH after 2 days	t-test significance with group	t-test significance p
A. 1.	None	7.11 ± 0.02		
2.	PTE Substitute	7.13 ± 0.04	1	N.S.
3.	PTH, 0.4 μg (1 unit)	6.92 ± 0.03	1	<0.001
4.	PTH, 2 μg (5 units)	6.89 ± 0.05	1	<0.001
5.	PTE, 1 unit	6.86 ± 0.03	2	<0.001
B. 1.	Acetic acid	7.07 ± 0.05		
2.	PTE substitute	7.08 ± 0.01	1	N.S.
3.	Peptide 3S, 0.4 μg	7.07 ± 0.04	1	N.S.
4.	Peptide 3S, 2 μg	7.07 ± 0.05	1	N.S.
5.	PTE, 1 unit	6.81 ± 0.01	2	<0.001
C. 1.	Acetic acid	7.03 ± 0.07		
2.	PTE substitute	7.06 ± 0.07	1	N.S.
3.	Peptide 2, 0.4 μg	7.02 ± 0.05	1	N.S.
4.	Peptide 2, 2 μg	7.06 ± 0.05	1	N.S.
5.	PTE, 1 unit	6.75 ± 0.04	2	<0.001
D. 1.	Acetic acid	7.22 ± 0.03		
2.	PTH, 0.4 μg (1 unit)	7.12 ± 0.01	1	<0.005
3.	Peptide 5, 0.4 μg	7.23 ± 0.06	1	N.S.
4.	Peptide 5, 2 μg	7.21 ± 0.03	1	N.S.

in this paper, for the simultaneous excretion of six lysosomal hydrolases (including acid phosphatase) during the development of osteoclastic bone resorption in tissue culture, they lead to the conclusion that at least part of the release of the enzymatic content of the lysosomes occurs at the very level where the first signs of bone resorption are visualized. As the whole content of the lysosomal bag is, in all probability, excreted in bulk, then not only the six acid hydrolases considered in this study, but also several others should be present at the level of the resorption zone. They would include, besides a nonspecific endopeptidase, cathepsin, an endomucopolysaccharidase, hyaluronidase (Vaes, 1967), and possibly also, although this is still a matter of debate (Gross, 1964), a collagenolytic enzyme which may be distinct from cathepsin, since there is evidence for the existence of such an enzyme in the granules (lysosomes?) of polymorphonuclear leucocytes (Lazarus et al., 1968) and possibly also in the lysosomes of bone (Woods and Nichols, 1965), of kidney (Schaub, 1964) and of liver cells (Wynn, 1967). All these enzymes could then depolymerize or merely, solubilize (Wynn, 1967), the macromolecular components of the organic matrix, thus allowing the osteoclasts to take up the resulting fragments by endocytosis to further digest them.

It should be clearly stated, however, that this hypothesis will remain largely speculative until it has been shown that the lysosomes of bone cells

TABLE X

Effect of PTE and of Purified PTH on the Release of Lactate and Citrate by Explanted Calvaria into their Culture Fluids

The calvaria were cultivated during 1, 2, or 3 days in 2 ml of medium containing 1 USP unit of PTE (experiment A) or of PTH (experiment B) per ml or appropriate amounts of their respective substitute solutions. Assays of citrate were done on pooled media from two cultures, while those of lactate were done on the individual culture fluids. There were 10 cultures per group in experiment A and 6 in experiment B.

Experiment	Medium assayed		mμMoles per calvarium	
			Lactate	Citrate
A	After 1 day	C	1613 ± 553	−6.3 ± 5.5
		PTE	2498 ± 400§	17 ± 13.5‡
	After 2 days	C	2274 ± 617	24.4 ± 16.8
		PTE	4875 ± 1025§	58.7 ± 35.6
	After 3 days	C	3266 ± 1824	12.7 ± 23.2
		PTE	6878 ± 1789§	92.8 ± 30.8§
B	After 1 day	C	1491 ± 492	13.2 ± 12.1
		PTH	2035 ± 232*	19.4 ± 6.4
	After 2 days	C	1990 ± 178	11.1 ± 14.6
		PTH	3200 ± 207§	47.9 ± 28.2
	After 3 days	C	2535 ± 612	−4.8 ± 10.4
		PTH	3918 ± 273§	89.2 ± 7.2§

*$p < 0.05$.
‡ $p < 0.01$.
§ $p < 0.005$.

indeed contain all the enzymes required to solubilize or to digest the various organic components of the bone matrix in the conditions (pH, ionic environment, etc.) prevailing in the resorption zones. The crucial problem of the nature, the mode of action, and the subcellular location of the collagenolytic enzymes active in bone resorption is extensively discussed elsewhere (Vaes, 1969); obviously, these points require further investigations if one wishes to draw a complete picture of the cellular mechanisms of bone resorption. It would be of importance to know if there exists in bone a collagenase similar to the enzyme which has been demonstrated and characterized by Gross et al. in some tissues of the tadpole (Gross and Lapière, 1962; Gross and Nagai, 1965; Nagai et al., 1966). Its existence would raise several questions: does it correspond or not to the collagenolytic factor which has been demonstrated in bone cells by Woods and Nichols (1965)? Is this enzyme of lysosomal or of other nature? Does it play a role, together with the lysosomal hydrolases, in the processes of the osteoclastic type of bone resorption, or is it active only in other forms of collagen degradation in bone, such as the osteocytic type of bone resorption or the destruction of newly formed collagen molecules during osteogenesis?

No precise mechanism can be proposed yet to explain the exocytosis phenomenon. PTE did not appear to have a direct disrupting action on the lysosomal membrane as it had no effect on the latency of β-glucuronidase when it was added directly to cytoplasmic extracts of bone cells; its behavior is different in this regard from that of vitamin A on cartilage (Fell and Dingle, 1963). The release of the lysosomal enzymes out of the cells is thus likely to result from a chain of events initiated by the hormone. Cyclic-AMP (adenosine-3′,5′[cyclic]-monophosphate) is probably a mediator of the action of PTH on bone resorption, as its dibutyryl-derivative duplicates the main actions of PTH on bone explants in culture: development of typical resorption lacunae, synthesis and exocytosis of lysosomal enzymes, stimulation of the release of lactate and citrate (Vaes,

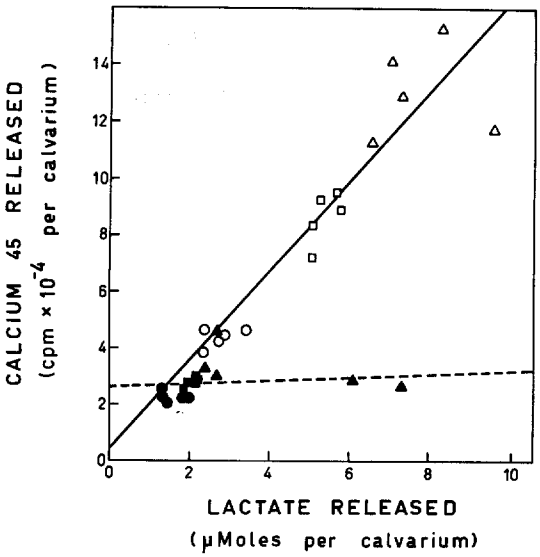

FIGURE 8 Correlation between the excretion of lactate and the progress of bone mineral resorption evaluated by the amount of ^{45}Ca released from labeled calvaria under the action of PTE. The explants were cultivated in 2 ml medium during 1 (circles), 2 (squares) or 3 (triangles) days, either without (full symbols) or with PTE (open symbols). For the PTE-treated cultures (solid regression line), $r = 0.922$; for the controls (broken regression line), $r = 0.168$.

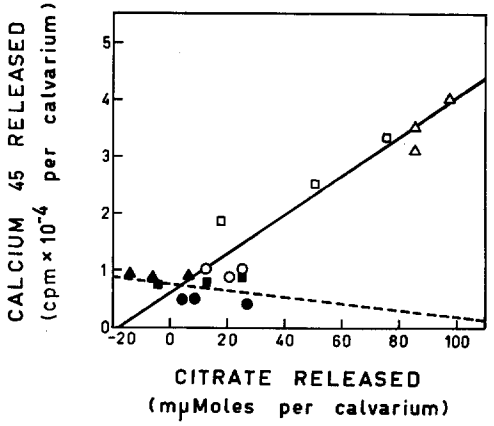

FIGURE 9 Correlation between the excretion of citrate and the progress of bone mineral resorption evaluated by the amount of ^{45}Ca released from labeled calvaria under the action of PTH. The explants were cultivated in 2 ml medium during 1 (circles), 2 (squares) or 3 (triangles) days, either without (full symbols) or with 0.4 μg of PTH per ml (open symbols). For the PTH-treated cultures (solid regression line), $r = 0.956$; for the controls (broken regression line), $r = -0.374$.

1968). However, according to preliminary experiments (Vaes, unpublished), cyclic-AMP and dibutyryl-cyclic-AMP, used alone or together with theophylline, do not cause the release of hydrolases from lysosomes in vitro. The increased local concentration of calcium, achieved through the solubilization of the mineral in the resorption zones, does not seem either to be a necessary link, as seen in this paper. A decrease in the calcium concentration of the culture fluid appeared even to stimulate the release of β-glucuronidase, but the significance of this observation, which has not been further investigated, is uncertain since it could be the result of toxic damage to the cells. We have indeed observed (unpublished observations) such an effect when various toxic chemicals (iodoacetate, Triton X-100) were added to the cultures or when the explants were cultivated in Hanks' solution, thus under conditions of starvation; however, in these cases, the intracellular stores of reference lysosomal enzymes (β-glucuronidase and acetyl-β-glucosaminidase) of the calvaria were quickly depleted and severe cell lesions were proven by an important reduction in the DNA content of the explants.

It is tempting to speculate (see Vaes, 1966 *b* and 1969) that the mechanism of exocytosis could

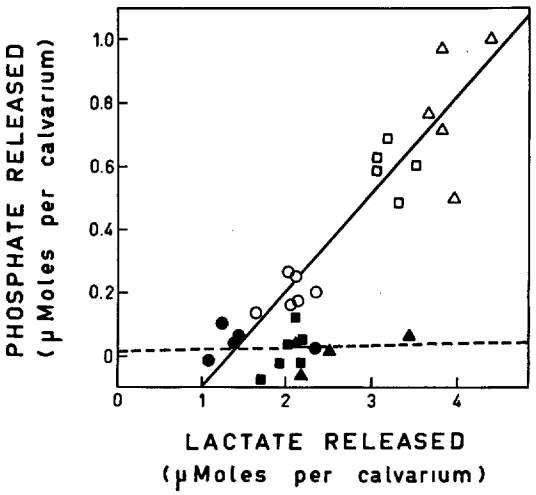

FIGURE 10 Correlation between the excretion of lactate and the progress of bone mineral resorption evaluated by the amount of inorganic phosphate released from explanted calvaria under the action of PTH. The experimental conditions and the symbols used are the same as on Fig. 9. For the PTH-treated cultures (solid regression line), $r = 0.901$; for the controls (broken regression line), $r = 0.0655$.

TABLE XI

Direct Comparison between the Release of Lactate, of Acid, and of Inorganic Phosphate by Calvaria Cultivated with or without Purified PTH

Calvaria, obtained from 18- or 19-day old mouse embryos, were cultivated during 45 hr in 2 ml medium 199 (+0.05 ml plasma, as usual). The media from four homologous cultures were then pooled and used for the measurement of pH and for the various assays; acid is expressed as μequivalents, lactate and phosphate, as μmoles released per calvarium over the 45 hr cultivation. The excess of acid required to bring the pH from the control medium to that of the corresponding PTH medium was measured by titration of a blank (medium cultivated without explant) with HCl. The fractions of phosphate ions present either as HPO_4^{--} or as $H_2PO_4^-$ were calculated by assuming a pK_2 value of 6.76 for H_3PO_4.

	C	PTH	PTH-C
A. 18-day old calvaria			
Lactate	1.15	2.11	0.96
pH	7.18	7.05	—
Excess of acid	—	—	0.50
Phosphate	−0.014	0.392	—
% as HPO_4^{--}	72.4	66	—
% as $H_2PO_4^-$	27.6	34	—
H^+ bound as phosphate ions	−0.018	0.525	0.543
B. 19-day old calvaria			
Lactate	1.47	3.09	1.62
pH	7.08	6.91	—
Excess of acid	—	—	0.52
Phosphate	0.092	0.732	—
% as HPO_4^{--}	67.6	58.5	—
% as $H_2PO_4^-$	32.4	41.5	—
H^+ bound as phosphate ions	0.122	1.036	0.914

be fundamentally similar to the mechanism through which the lysosomal content is secreted into the endocytosis vacuoles or phagosomes (for a review, see de Duve and Wattiaux, 1966). The ultrastructural observations of Scott (1967) indeed support the view of a direct excretion of the lysosomal content into the closed vacuoles or phagosomes of the osteoclast ruffled border (which contain acid phosphatase and β-glucuronidase, as shown by Hancox and Boothroyd, 1963, and by Doty and Robinson, 1968) and also into the numerous neighboring channels or saccules which are still in direct communication with the extracellular resorption zone underneath the brush border (for illustration of these structures, see Hancox and Boothroyd, 1963).

Synthesis of Lysosomal Enzymes

Several experiments have shown an important increase in the activity of various lysosomal hydrolases recovered from the explants and from their culture fluid during the development of the resorption process. There seems to be little doubt that this is due to new synthesis of enzyme, as it is unlikely that several different hydrolases, preexisting in the tissue, would be simultaneously activated by chemical modifications of their environment accompanying the resorption process. Moreover, the experiments done with the use of actinomycin D and puromycin support the interpretation that an increased synthesis of protein is necessary for the development of bone resorption (Gaillard, 1965; Raisz, 1965) since these inhibitors prevented the appearance of resorption lacunae in the tissue; they inhibited simultaneously the synthesis and the excretion of β-glucuronidase, the reference acid hydrolase. These drugs are, however, toxic to many metabolic processes in the cells, and caution is required in the interpretation of their effects. However, recent radio-

autographic data (Owen and Bingham, 1968) show that PTH stimulates the synthesis first of nuclear and later of cytoplasmic RNA in osteoclasts (while decreasing it in osteoblasts) and it is tempting to speculate that this may be linked to the synthesis of lysosomal enzymes in our resorbing explants.

Through this increased synthesis, the stores of acid hydrolases could be maintained in the cells even in the presence of a continuous excretion of these enzymes; at the beginning of the cultures (see Table V, day 2), there may however exist some depletion of these intracellular stores which is only compensated later.

The mechanism controlling this increased synthesis is unknown; it could result from a direct activation of the genes by PTH, or from an increased pinocytosis in the osteolytic cells, as suggested by the observations of Cohn and Benson (1965) on macrophages in culture, or from an increased permeability of the cells to calcium ions, as suggested by Park and Talmage (1968).

Excretion of Acid and Resorption of the Mineral

PTE, as well as pure PTH, stimulated the rate of acid release by the calvaria in culture, as demonstrated by the faster acidification of the media bathing the explants. It also enhanced the release of lactate: this suggests that the excess acid may originate from a stimulation of the aerobic glycolysis of the explants. Time-course relationships, as well as the good correlations established over the first 3 days of culture (a period over which the calvaria have lost half of their mineral content) between the release of lactate by the cells and that of ^{45}Ca or of inorganic phosphate from the mineral in the PTE of PTH-treated explants, are compatible with a causal role of the glycolysis in the solubilization of the mineral. The acid produced could act by converting an insoluble salt of calcium and phosphate to soluble ions, following a reaction of type: $Ca_3(PO_4)_2 + 2\ H^+ \rightarrow 3\ Ca^{++} + 2\ HPO_4^{--}$, or, in the case of fully formed hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2 + 8\ H^+ \rightarrow 10\ Ca^{++} + 6\ HPO_4^{--} + 2\ H_2O$. Both the phosphate released by resorption from the mineral and the excess of acid produced under PTH can almost be accounted for by the excess of lactate which is simultaneously released by the PTH-treated explants, providing that this release of lactate is accompanied by the release of an equimolar amount of H^+ ions (Table XI).

The lack of correlation between the release of lactate and that of ^{45}Ca or of inorganic phosphate in the control cultures, is not surprising. The explants have indeed a mixed cell population containing, besides a few osteoclasts, a majority of osteoblasts and of osteoprogenitor cells which are not directly active in resorbing bone and which separate the mineral from the culture fluid by a continuous protective layer. It is known that normal bone tissue (for a review, see Vaes, 1966 *b*) and isolated bone cells, apparently mainly osteoblasts (Peck et al., 1964), have a high rate of aerobic glycolysis. Presumably, the acid produced in this process by the nonosteolytic cells is excreted directly in the culture fluid without even being in contact with the mineral (and any change in this component of the total acid production of the tissue will not affect the solubilization of bone salts); osteolytic cells only would then release acid at the surface of the mineral, a hypothesis which is supported by some limited evidence suggesting that the pH of the resorption zones underlying the osteoclasts, is acid (Cretin, 1951; Neuman et al., 1960).

Although clearly stimulated by PTE or by PTH and also in good correlation with the mobilization of the mineral from the resorbing explants, the release of citrate seems to be insufficient, on a molar basis, to explain this resorption, even if its calcium chelation property is taken into account together with the acid which presumably originates during its formation; this insufficiency has already been noted in short-term metabolic studies on PTE-treated bones (Borle et al., 1960; Vaes and Nichols, 1961 and 1962). However, the possibility should be considered that some of the citrate released from the tissue into the culture fluid has been consumed by the cells and thus, that the measurement of the amounts of citrate present in the medium underestimates the quantities released from the cells. Yet PTH is known to decrease the uptake and the oxidation of exogenous citrate by bone cells (Cohn and Griffith, 1965; Martin et al., 1965; for a review, see Vaes, 1966 *b*), so that the excess of citrate found in the culture fluids of PTH-treated explants, as compared to the controls, could then merely reflect this difference of uptake and still have no relationships with the solubilization of the mineral in the resorption zones, which depends on the release of mineral-solubilizing

factors by the resorbing cells. Moreover, as discussed elsewhere (Vaes, 1966 *b*), the release of citrate could be a consequence rather than a cause of the release of the mineral as it could be due to the inhibition of the enzyme aconitase in the cells by an increase in the intracellular concentration of calcium (Hekkelman, 1963).

Furthermore, if a secretion of a calcium-chelating agent in the resorption zones were the cause of the solubilization of the mineral, this phenomenon should be accompanied by a progressive alkalinization of the medium, owing to the release of PO_4^{---} ions and their transformation into HPO_4^{--} or $H_2PO_4^{-}$ ions; instead, we observed an acidification. On the other hand, the smaller activity of alkaline phosphatase in the medium bathing the resorbing explants and the progressive depletion of the PTE-treated tissues in this enzyme does not support a hypothetical causal role of this hydrolase (Rasmussen and Tenenhouse, 1967) in the mobilization of bone mineral in the present culture system.

PTH itself was responsible for the release of acid; other nonhormonal peptides, isolated from the parathyroid gland by Rasmussen et al. (1964) and by Hawker et al. (1966) were inoperant. This contrast with the observations made by the authors on the effect of some of these peptides on the aerobic glycolysis of ascites Ehrlich tumor cells (no effect of the hormonal peptide, PTH, but stimulation by the nonhormonal peptide 3S); thus the conclusions drawn from these observations in tumor cells (see also Tenenhouse et al., 1966) cannot be extrapolated as such to the action of PTH in bone resorption. The lack of effect of PTE on the lactate production by rat fibroblasts in culture also underscores the danger of such extrapolations.

The mechanism through which acid is being released into the resorption zones could involve

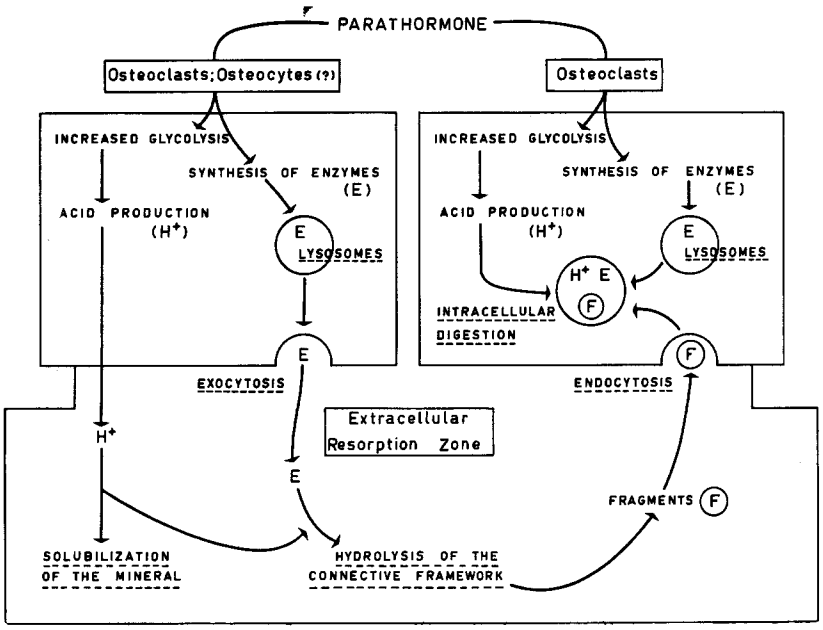

FIGURE 11 A working hypothesis concerning the cellular mechanism of bone resorption. According to this view, the acid hydrolases of the lysosomes are excreted in bulk, through exocytosis, in the extracellular resorption zone underneath the ruffled border of the osteoclasts (maybe also around the osteocytes), where they exert a concerted eroding action on the organic components of bone matrix; the phenomenon can perdure thanks to a new synthesis of lysosomal enzymes which is stimulated by PTH in the cells. The simultaneous excretion of acid in the resorption zones, made possible mainly through a stimulation of aerobic glycolysis by PTH, will allow the solubilization of the mineral component of the matrix and favor at the same time the action of the acid hydrolases. Fragments released from the matrix by this extracellular resorption may be taken up in the osteoclasts through pinocytosis, to be further digested intracellularly by the lytic action of the same agents, lysosomal hydrolases and H^+ ions.

active transport phenomena by the plasma membrane of the osteoclasts. It could be similar to the mechanism which is presumed (see de Duve and Wattiaux, 1966) to operate to acidify the content of the nearby digestive vacuoles of the ruffled border.

A General Working Hypothesis

In conclusion, a working hypothesis may be presented to explain at the biochemical and cytological levels the action of osteoclasts in bone resorption (Fig. 11); this view is further discussed elsewhere (Vaes, 1966 *b* and 1969). How far this hypothesis may be extended to nonosteoclastic forms of bone resorption (Belanger et al., 1963; Cameron et al., 1967) remains to be elucidated. It would be of importance, indeed, to know if lysosomal hydrolases and acid may be considered as the agents of all forms of bone mobilization or if other agents and mechanisms, such as the making and the excretion of a specific, nonlysosomal, collagenase (Gross, 1964) or that of a pyrophosphatase active in the mobilization of the mineral (Fleisch et al., 1966) may be involved in some forms of bone resorption. Also, when considered in the light of the action of PTH on bone, it should be kept in mind that the stimulation of osteoclastic bone resorption constitutes only one aspect of this action and that the other aspect, the transfer of calcium from bone to the extracellular fluids to maintain calcium homeostasis, may be dependent on completely different mechanisms (Talmage, 1967).

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