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ALVEOLAR BONE RESORPTION AFTER CALCIUM DEFICIENCY IN RATS

BY

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ABSTRACT

The purpose of this experiment was to produce a progressive alveolar bone resorption due to calcium-deficient diet and to investigate the subsequent morphological changes of the bone cells. One hundred and twenty-seven Wistar rats aged 35 days were maintained for 8 weeks on a diet containing either 0.6106% calcium (control group) or 0.0098% calcium (experimental group), both diets being the same in phosphorus content (0.3867%) with adequate vitamin D. The animals of both groups were sacrificed at intervals of two weeks each. Ten animals of the experimental group were replaced with the control diet at the sixth week to see the curative effect on alveolar bone resorption. Serum calcium level in the experimental group was slightly but significantly lower than that of the control. Serum phosphorus level was not different from that of the control group. Severe alveolar bone resorption accompanied with both the indentation from endosteal surfaces and osteocytic osteolysis from the inner site of bone was ascertained by microradiographic examinations. Histological findings of decalcified sections suggested that a number of hypertrophied endosteal cells, not osteoclasts, were attaching to the indentations. These hypertrophied cells, however, were hardly found in the recovered animals. The relation between these phenomena and parathyroid function was discussed.

INTRODUCTION

It has previously been shown in a number of animal experiments that a diet deficient in calcium will produce osteoporosis (Harrison and Fraser[1]; Scott *et al.*[2]; Jowsey and Gershon-Cohen[3]) rather than osteomalacia, provided the levels of vitamin D are adequate. There is a reduced amount of bone present in the skeleton; the ash content of such a bone is normal, and osteoid borders are small or absent (Nordin[4,5]). These reports are mostly on the growing epiphyseal bone. There are, however, a few reports on alveolar bone.

The purpose of the present investigation was to produce a progressive bone resorption in rats alveolus by means of a calcium-deficient diet, and to

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see in detail the subsequent changes of the bone cells which are taking part in bone formation and resorption. Serum calcium and phosphorus levels were also measured to examine the participation of parathyroid gland with the destruction mechanism of the bone.

Materials and Methods

One hundred and twenty-seven male rats of the Wistar strain, aged 35 days, were used in this experiment. Eleven of these animals were sacrificed at the beginning of the experiment as 0-week rats. Fifty-three animals were fed on a control diet and 63 animals were fed on a calcium-deficient diet. Ten animals of the latter group were transferred to the control diet after 6 weeks of the experiment and reared for further 2 weeks to see the curative effect.

The calcium-deficient diet consisted of corn starch 66.2724 g, lactic casein 18.0000 g, cotton seed oil 10.0000 g, salt mixture[6] 4.9530 g, and vitamin mixture[7] 0.7746 g (per 100.0000 g of diet). The salt mixture was composed of KCl 1.0000 g, $NaHCO_3$ 0.7000 g, $Fe_2(SO_4)(NH_4)_2SO_4 24H_2O$ 0.5080 g, KH_2PO_4 1.7000 g, NaCl 0.5000 g, and $MgSO_4 7H_2O$ 0.5450 g. The composition of the vitamin mixture was as follows (mg/100.0000 g of diet): choline chloride 400.0, inositol 250.0, p-aminobenzoic acid 100.0, nicotinic acid 5.0, α-tocopherol 2.5, 2-methyl-1,4-naphthoquinone 3.0, pyrodoxine hydrochloride 1.2, riboflavin 1.6, thiamine hydrochloride 0.8, folic acid 0.5, vitamin B_{12} 0.006, viosterol 432.0 IU, and vitamin A 10.0. The control diet has the same general composition, except that 1.5010 g/100.0000 g of corn starch was replaced by 1.50000 g of $CaCO_3$ and 1.0 mg of calcium pantothenate.

The net content of calcium[8] in each diet was checked by atomic absorption spectrophotometry after the materials were wet ashed in chloric acid[9]. The percentage of calcium content was 0.6106% in the control diet and 0.0098% in the calcium-deficient diet. The total phosphorus content was the same for both diets and calculated to be 0.3867%. All the animals were given deionized water freely. All the 116 animals were sacrificed at intervals of 2 weeks throughout the whole experimental period lasting for 8 weeks.

The blood was taken from the carotid artery under ether anesthesia for the determination of serum calcium and phosphorus levels. The serum calcium[10] was measured by using an atomic absorption spectrophotometer (Model 303, Perkin-Elmer Ltd., U.S.A.) and serum phosphorus[11] was measured by a photoelectric spectrophotometer (Model EPB-U, Hitachi Ltd., Japan).

Both sides of the mandibular bones were removed and cleaned of muscle and soft tissue. Radiographs of the mandibular bones of each animal were immediately taken by using a Softex EMB-type apparatus (Softex Co., Ltd., Japan).

The right-side bones were fixed in 10% neutral Formalin, dehydrated in ethanol, and embedded in Rigolac (Riken Polyester Resin). Buccoligual sections of the mandible were cut in 90 μ in thickness by using a Bronwill Thin Sectioning Machine (Bronwill Scientific Inc., U.S.A.). The contact microradiographs of the sections were taken by a Softex CMR-type apparatus (Softex Co., Ltd., Japan) to see the degree of alveolar bone calcification. The conditions were as follows: exposed for about 9 minutes with Eastman Kodak 649-0 spectroscopic film at 100 V, 3.0 mA, developed in Kodak D-19 and fixed in Kodak fixer.

Other mandibles were fixed in 10% neutral Formalin at pH 6.9 and decalcified with 10% EDTA, embedded in paraffin, and cut transversely in 4 μ thickness. Serial paraffin sections of decalcified specimens were stained with Hematoxylin and Eosin.

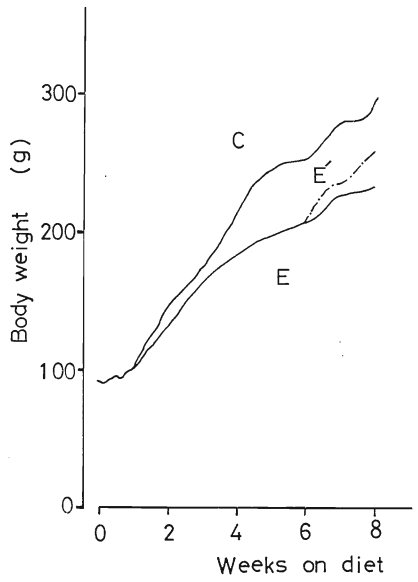


Fig. 1. Body weight changes during 8 weeks of experiment in control animals (C), calcium-deficient animals (E), and animals replaced on the contral diet after 6 weeks of calcium deficiency (E′).

Results

Growth

The difference in weight gain between the experimental and control groups became pronounced with time (Fig. 1). When the animals were transferred to the control diet after 6 weeks, recovery was hastened. The amount of food consumption returned to the normal level and the weight gained increasingly. No tetany was seen during the whole experimental period.

Serum calcium and phosphorus levels

The serum calcium levels of the experimental animals in 2- to 8-week groups were slightly lower than those of the control group and the t-test supported the conclusion of significant difference ($p<0.01$). The serum calcium level started to fall slowly after the second week; the mean and standard deviation of the serum calcium level in the control group was 9.78 ± 0.30 mg% whereas that of the experimental group was 9.39 ± 0.28 mg%. At the eighth week of experiment serum calcium reached the lowest level of

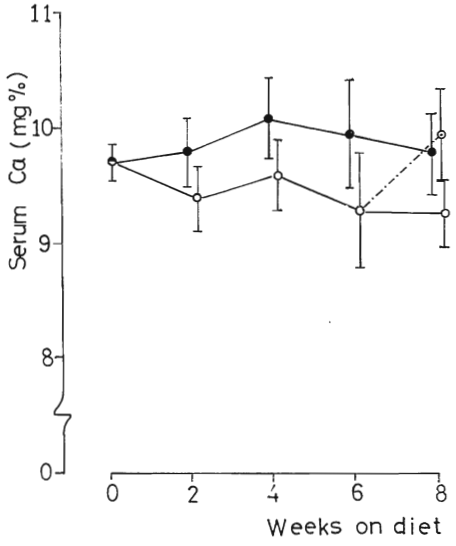


Fig. 2. Serum calcium level during 8 weeks of the experiment. ●——● control group, ○——○ calcium-deficient group, ⊙-·-·⊙ the animals placed on the control diet at the sixth week of the experimental period and fed for further 2 weeks. Vertical bars represent standard deviation of the mean.

9.26±0.30 mg%. The serum calcium in the animals which were changed to the control diet from 6 weeks after the beginning of the experiment, however, was 9.94±0.39 mg% and no significant difference between this group and control group was ascertained ($p>0.05$) (Fig. 2).

The serum phosphorus levels in both the control and the experimental groups became lower with time (Fig. 3). Change of the diet seemed to have no effect.

Radiograms of the mandible

The radio-opacity of the bone around the molars of the calcium-deficient rats decreased gradually as the experiment progressed. Lamina dura was poorly defined from 2 weeks on the calcium-deficient diet and the lamina was no longer visible in the radiogram from 4 weeks on. The overall radio-opacity of the remaining alveolar bone also decreased markedly, and it was very striking at the eighth week (Fig. 4).

When the experimental animals were transferred to the control diet at

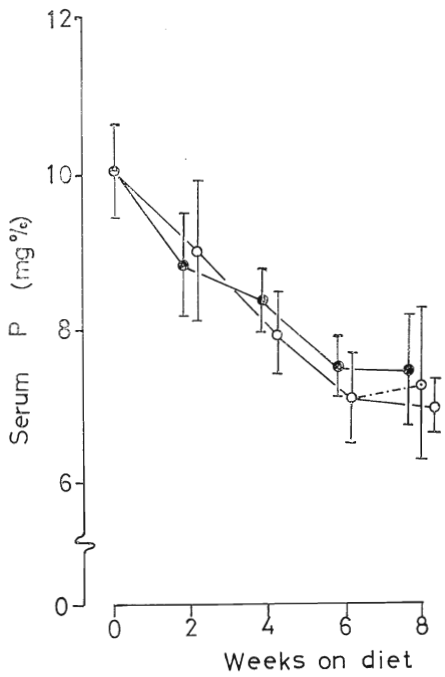


Fig. 3. Serum phosphorus level during 8 weeks of the experiment. ●——● control group, ○——○ calcium-deficient group, ⊙-·-·⊙ group of animals fed the control diet for 2 weeks after 6 weeks on calcium-deficient diet. Vertical bars represent standard deviation of the mean.

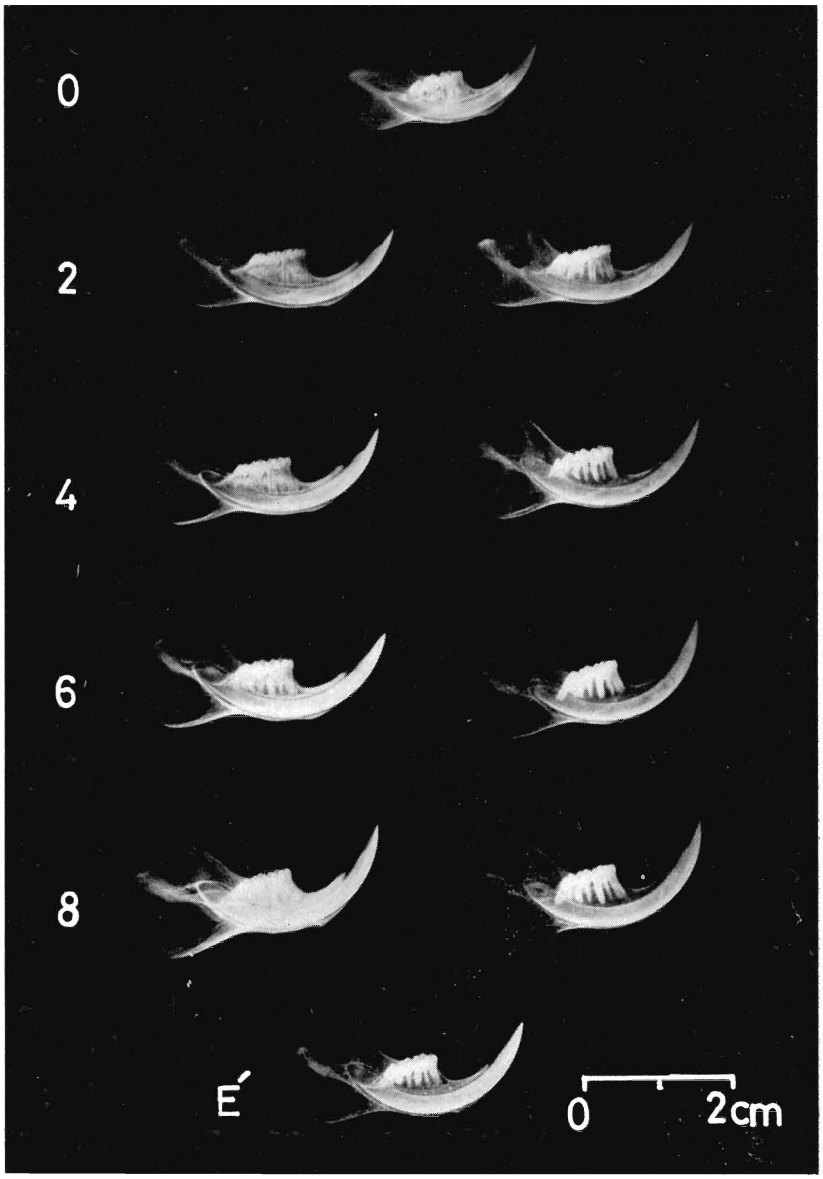


Fig. 4. Radiograms of rat mandibles. Left row, control animals; right row, calcium-deficient animals. 0, 2, 4, 6, and 8 show the experimental period in weeks. E′ shows the mandible of the animal placed on the control diet for 2 weeks after 6 weeks of experimental diet.

the sixth week, however, the alveolar bone appeared to increase in radio-opacity.

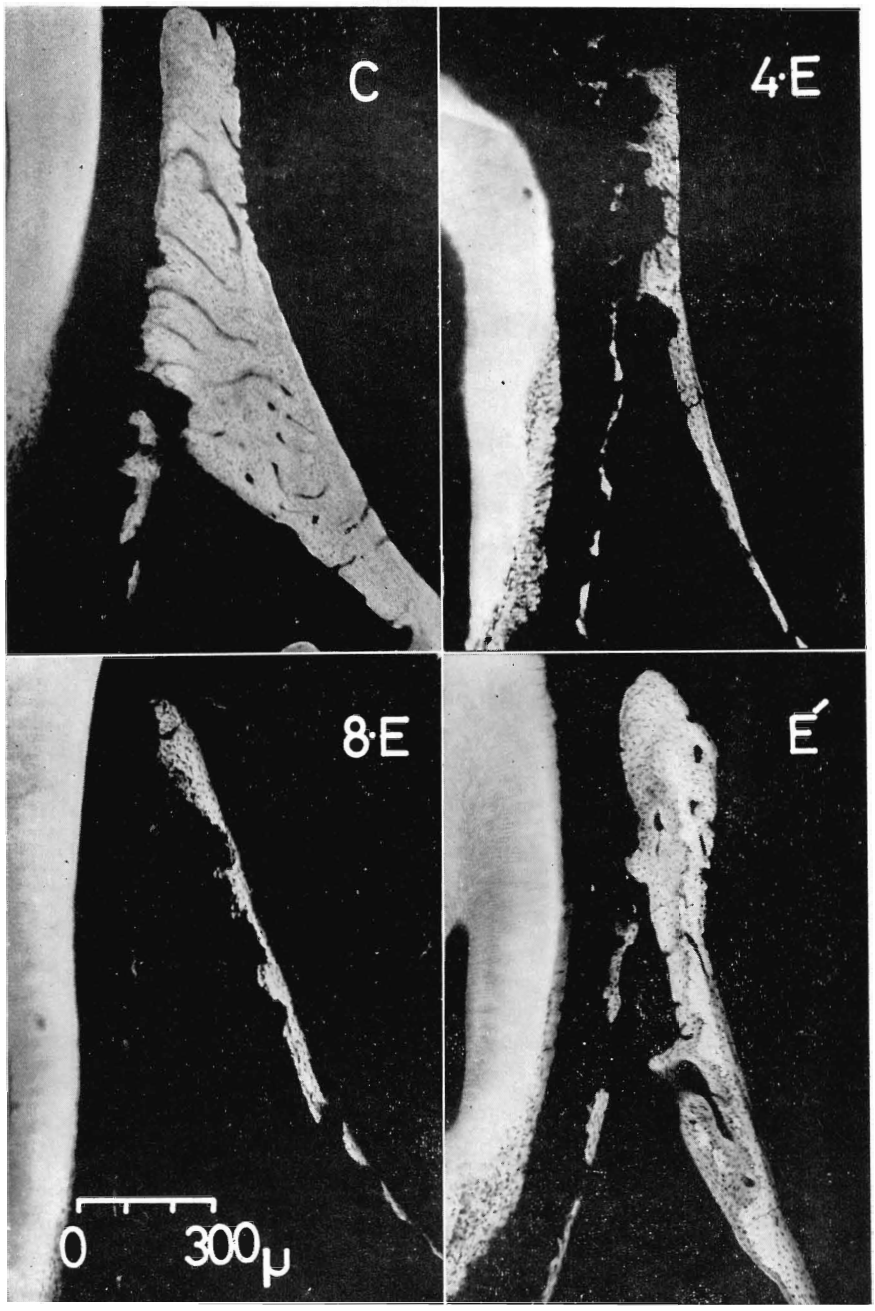
Microradiograms of alveolar bone

Microradiograms of ground sections confirmed the results of radiographic appearances with regard to progressive changes in the alveolar bones. In animals on the calcium-deficient diet for 8 weeks, the contours of the periosteal alveolar wall were smooth and the height of the crest seemed to be not so reduced as compared with the controls. The progressive bone resorptions were found remarkably on the endosteal areas of the alveolar bone with striking indentations which were suggestive of Howship's lacunae. They were most pronounced in the *alveolar bone proper* facing the tooth root with very thinning of the compact bone. These findings were observed to a slight degree even in the rats on a calcium-deficient diet for 4 weeks. The remaining bone became thinner, and radiolucent areas with enlarged osteocytic lacunae were found. The frequent confluences of the adjacent lacunae which must be interpreted as evidence of osteolysis were occasionally obvious. These findings may indicate that the alveolar bone resorption was derived from both osteolysis of osteocytes seated deeply in the bone tissue and osteoclasis induced by accelerating cavity formation at the endosteal surface of the compact bone (Fig. 5).

When the calcium-deficient rats were placed on the control diet, restorative appearance in the microradiogram was very striking. New bone formation was observed at the endosteal surface of the alveolar compact bones. Many indentations of the endosteal surface disappeared and the surface returned to a normal appearance with smooth outline. The thickness of the alveolar bone was restored slightly but radio-opacity of the newly formed bone was somewhat lower than that of the old bone. Enlarged or confluent osteocytic lacunae were rarely seen in the old bone tissue.

Histological changes in decalcified sections

Histological findings on decalcified sections revealed distinguished difference in the alveolar bones between the control and the calcium-deficient rats. The differences were especially well observed in the endosteal surfaces where the indented resorption was shown in the microradiograms. In the experimental animals, a number of hypertrophied endosteal cells appeared in these resorption areas (Fig. 6). They resembled hypertrophied osteoblasts in morphological features. An osteoid was not present in these portions. A few of osteoclasts, characteristic of multinucleated giant cells with eosinophilic stain, were seen in some resorptive cavities but they were still observed, to some extent, even in the control bones. The nuclei of the osteocytes found in the remaining bone tissue were rather large in size and


C
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stained more weakly to Hematoxylin than those of the control. These cells embedded in somewhat loosened bone matrix were arranged in a disorderly fashion (Fig. 7).

Only after two weeks' restoration on the control diet, the hypertrophied osteoblastic cells in features disappeared from the endosteal surface. New bone formation by the typical shape of osteoblasts was noted and the endosteal surface regained a smooth outline. Even under the process of these rapid recoveries, a few osteoclasts were rarely seen on the edge of the old bone, as well as in the control. The osteocytes in the old bone ceased to be hypertrophic and were fairly uniform in size.

Discussion

It has been well known that a diet deficient in calcium but adequate with vitamin D elicits osteoporosis in animals[1-3] (Ferguson and Hartles[12]). Osteoporosis causes the removal of minerals from the bone followed at once by the removal of matrix, (Cartter *et al.*[13]), thus reducing bone mass without reducing the mineral content of the residual bone (Nordin[4]).

In the present experiment, an excessive alveolar bone resorption due to osteoporosis was observed in rats. It was of much interest that serum calcium level slightly but significantly lowered whereas that of phosphorus was not significantly different from that of the control group. Early investigators[2,3] reported normal serum calcium levels, hyperplasia of parathyroid glands, and appearances of osteoclasts[2] during calcium deficiency and therefore, they seemed to be convinced that calcium deficiency in animals would stimulate the activity of parathyroids to maintain blood calcium homeostasis. On the other hand, Crawford *et al.*[14] have shown that, if vitamin D is supplied, low calcium diet does not produce parathyroid hyperplasia. They reported that the serum calcium level decreased significantly but serum inorganic phosphorus level was not different from that of the control group. According to Henrikson[15], serum calcium level remains within normal limits but serum phosphorus level decreases in dogs fed on a low-calcium diet, with a phosphorus content three times greater than the control diet. He reported that maintenance of isocalcemia reflected the action of para-

Fig. 5. Microradiograms of the lingual alveolar bone of bucco-lingual sections. C: control rat in 8th week, 4–E: experimental animal in 4th week, 8–E: experimental animal in 8th week, E′: animal transferred to the control diet after 6 weeks of experiment. In 4–E and 8–E, indentations are conspicuous at the endosteal surface. Enlarged osteocytic lacunae and confluences of adjacent lacunae are prominent. The remaining bone becomes thinner. In E′, new bone formation is shown with a less increased radio-opacity than that of the old bone. The endosteal surface with smooth outline is observed.

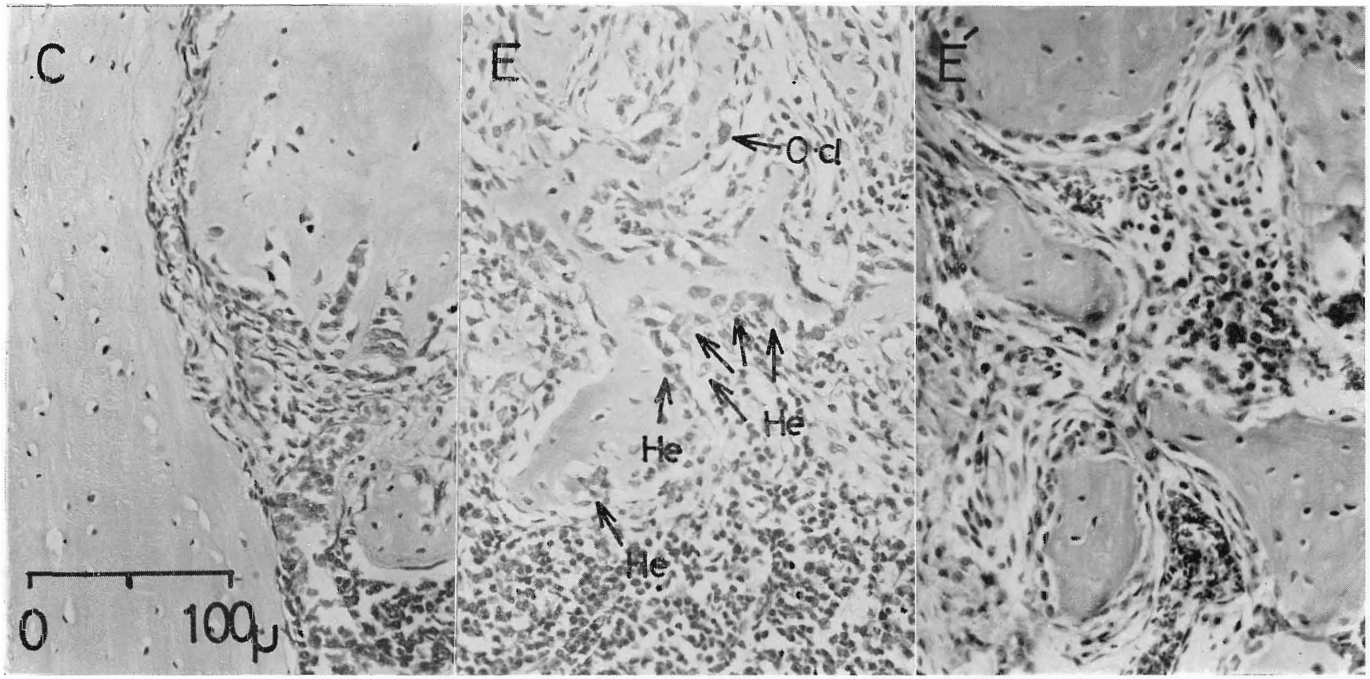

Fig. 6. Photomicrographs of decalcified bucco-lingual sections. Hematoxylin and Eosin stain.
C: control rat in 8th week.
E: calcium-deficient animal in 8th week.
E′: animals replaced on the control diet for 2 weeks after 6 weeks of the experimental diet.
He: hypertrophied endosteal cell. O·cl: osteoclast. In E, a number of hypertrophied endosteal cells (He) appear in the resorption area. In E′, only after 2 weeks of restoration on the control diet, the hypertrophied osteoblastic cells in features disappeared from the endosteal surface. New bone formation by the typical shapes of osteoblasts is very prominent and the endosteal surface seems to regain smooth outlines.

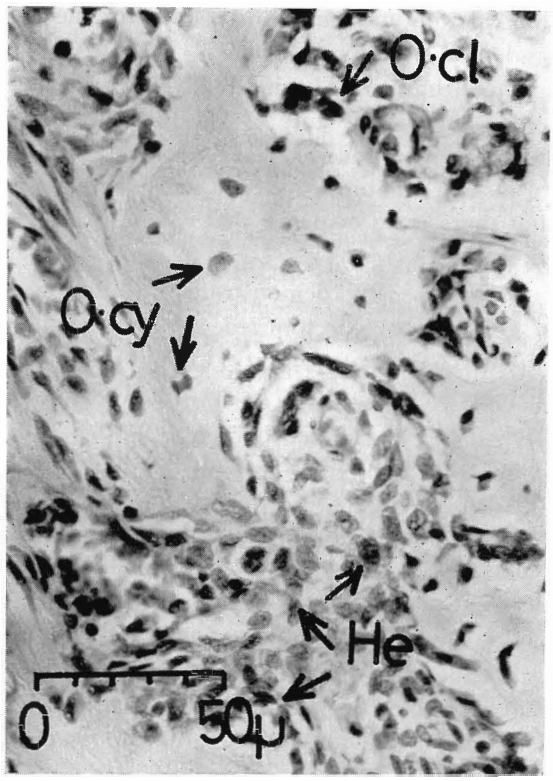

Fig. 7. Photomicrograph of resorption site of an animal fed on the calcium-deficient diet (high power magnification). It is observed that a number of hypertrophied endosteal cells (He) appeared in these resorption areas. They resemble hypertrophied osteoblasts in morphological features. The nuclei of the osteocytes (O·cy) found in the remaining bone tissue are larger and stained more weakly to Hematoxylin than those of the control. A few of the osteoclasts (O·cl) are seen in some resorptive cavities. But they are still observed, to some extent, even in the control bones.

thormone and serum phosphorus decreased continuously because of the action of parthormone on the kidney, and that still better clinico-pathological evidence for secondary hyperparathyroidism was furnished by serum phosphorus determination. His views, therefore, seemed to define the osteoporosis due to low-calcium diet as the state of secondary hyperparathyroidism in spite of no appearance of hypertrophied parathyroid glands.

If serum levels of both calcium and phosphorus are available as an index of the parathyroid function, as shown by Henrikson, the results ob-

tained on serum calcium and phosphorus levels in the present experiment may be independent of parathyroid glands. Nordin[4] supported Crawford's view and commented that it had been already pointed out that tissue minerals were presumed to be in a state of equilibrium with the crystals of a calcium in phosphate compound in the bones. He states also that the maintenance of plasma calcium is independent of the degree of parathyroid activity and that there is no evidence that calcium deficiency *per se* stimulates the parathyroids.

Recently, Jowsey *et al.*[17] demonstrated in their figure that heparin administration to parathyroidectomized kittens elevated serum calcium values from about 5.5 mg% to about 11.5 mg% and suggested that the calcium mobilizing effect of heparin was a direct one. So they stated that their observation, made *in vivo,* alluded a somewhat different mechanism from that proposed by Goldhaber[18], namely, that heparin enhanced the action of parathyroid hormone.

Histological appearance of excessive alveolar bone resorption due to calcium deficiency may suggest the two facets of bone resorption. One of these might be taken as the resorption from inner site of bone with osteocytes and the other might be the resorption from outer endosteal surface. It seems likely that they concomitantly dissolve the bone mineral and bring out the matrix at once (Nordin[4]; Talmage and Elliott[19]). The findings in the decalcified sections and the microradiograms indicate that the inner site of bone resorption was induced by osteocytic osteolysis. Osteocytic osteolysis which has been formulated by Bélanger[20,21] was observed even in the present experiment.

The other bone resorption from the endosteal surface was clearly observed on the microradiograms as a serial of indentations. A number of osteoclasts had been expected to be in the cave of these indentations as the course of experiment progressed but the number of osteoclasts seemed not to increase. There were found only a few osteoclasts in all the three groups. It seemed difficult to accept that such a few osteoclasts performed these excessive bone resorptions. It can be argued, according to the relatively short life-span of the osteoclasts, that their absence in the section would not preclude osteoclasia (Hancox[22]). Nevertheless, if osteoclasia played any decisive role in the resorption of alveolar bone in the present experiment, it would have been demonstrated more often than was the case.

The major histological change in the remaining bone was increased number of hypertrophied endosteal cells. It is unlikely that this increase was due to a new matrix formation or its calcification, because there could not be found osteoid seams but excessive bone resorption on the microradiograms. When the animals were transferred to the control diet after

6 weeks of calcium deficiency, much of these hypertrophied cells disappeared and new bone formation by a number of typical osteoblasts was observed. These findings may suggest that the hypertrophied endosteal cells have an osteoclastic activity. It may be reasonable to expect that these cells can acquire osteogenic activity again when the environment was corrected by restoration of calcium. McLean *et al.*[23] observed that osteoblasts were changed to spindle cells, phagocytes, osteocytes, and osteoclasts by parathyroid extract. Tonna[24] also postulated that rapid changes in osteoclastic cell population were explained on the basis of formation of osteoclasts from osteoblasts and the dissociation of osteoclasts into precursor cells.

Recently, Salomon[25] found hypertrophied cells on the endosteal surfaces of calcium-deficient rats and suggested that it would appear that one and the same cell may either form the bone when calcium supplies are adequate, or remove the bone as in the earlier stages of calcium deficiency. These concepts are consistent with the present experiment, but Salomon seems to think that these cells are derived from osteocytic osteolysis under the influence of increased parathyroid activity. It is a problem requiring further investigations whether experimental osteoporosis is induced by the secondary hyperparathyroidism and whether cell transformation is in response to environmental change or not.

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