OSTEOSCLEROSIS AFTER INTERMITTENT ADMINISTRATION OF LARGE DOES OF VITAMIN D IN THE RAT

A Note on the Pathogenesis of Osteopetrosis

E. STOREY, MELBOURNE, AUSTRALIA

From the Department of Pathology, University of Melbourne

The rate and amount of growth of any tissue are dependent on a number of stimuli, of which some are known but others are not so well understood. Differences in the rate of growth are demonstrated more easily in bone than in most other tissues, and it is clear that when the stimuli are sustained the growth and remodelling of bone are continuous, but when the stimuli are interrupted or discontinuous, growth and remodelling are intermittent. This is well shown in some animals by bone changes which recur naturally at regular intervals; that is, they are cyclical in nature and appear to be under hormonal control. These cycles may be seasonal—for example, egg laying of pigeons (Bloom, Bloom and McLean 1941), moulting birds (Meister 1951), growth of antlers in deer; or daily, as in laying hens (Bloom, Domn, Nalbandov and Bloom 1958). Similar cyclical changes may be induced artificially in the rabbit by intermittent administration of hormone (Storey 1958).

The osseous structure in some bone diseases also suggests that the condition is essentially a cyclical disturbance of the normal growth and remodelling of bone. Some of the osteodystrophies, such as osteitis deformans and osteopetrosis, may be explained in this way, as manifestations or modifications of a cyclical process. This is especially obvious in osteopetrosis, in which transverse bands at sites of endochondral bone growth compel attention. Previous experiments have shown that bone changes produced respectively by continuous or intermittent administration of cortisone are different and range from porosis to mosaics of bone deposition (Storey 1958).

In the present work bone changes associated with intermittent administration of vitamin D were investigated. Experiments with continuous large doses of this vitamin have shown only the formation of osteoid tissue with delayed calcification—the condition known as "hypervitaminosis D rickets" (Ham and Lewis 1934). When it is administered intermittently remodelling of bone does not occur normally: more bone is laid down than is resorbed, and therefore bones become denser than normal; calcification, although delayed, occurs and bones become harder and brittle. Increase in the length of time between doses of vitamin D is associated with extremely variable bone changes ranging from uniformly dense bone, through transverse bands in the metaphysis to dense remnants in the medullary cavity. Bone changes in some respects resembling those of osteopetrosis are produced by intermittent vitamin D administration, and they form the basis of the present study.

REVIEW OF OSTEOPETROSIS

Reviews of various aspects of osteopetrosis are available (McCune and Bradley 1934, Zawisch 1947, Pines and Lederer 1947, Fairbank 1948); to avoid repetition only papers relevant to the present problem will be discussed. The disease was first described by Albers-Schönberg (1904) and, because of the petrified nature of the bones, was termed osteopetrosis by Karshner (1926); at the same time, in different subjects bones have been described as chalk-like (Pirie 1930), hard and brittle (Pines and Lederer 1947), or both soft and hard in different bones of the same patient (Enticknap 1954). Radiographs show that, throughout the skeleton, bone is abnormal in density and amount; in severe examples the marrow cavities are completely obliterated. One feature usually commented upon and referred to as "pathognomonic of osteopetrosis" is an alternation of radio-opaque and radiolucent transverse
bands running parallel to the epiphysial cartilage of the long bones and to the surface of other bones, particularly the iliac crest (Ellis 1934, Pines and Lederer 1947, Fairbank 1948, Cohen 1951, Callender and Miyakawa 1953). In the long bones these have been interpreted as healed fissure fractures (Cohen 1951), or signs of intermittency of endochondral growth (Lamb and Jackson 1938, Zawisch 1947). In addition to transverse bands, which suggest a periodical remission of the disease (Weinmann and Sicher 1955), longitudinal striations (Fairbank 1948) or widening of epiphysial lines—interpreted as signs of slight to gross rickets—may be present (Nussey 1938; Pincus, Gittleman and Kramer 1947).

Histological studies have led to general agreement that bone is both increased in amount and abnormal (woven rather than lamellar) in structure. All stages of growth may be found, from young unremodelled bone onwards. Areas of normal radiodensity show histologically normal bone (Pines and Lederer 1947). Islands of densely calcified cartilage occur near the epiphysis enclosed in masses of woven bone, the lamellar arrangement of collagen fibres being absent (Engfeldt, Engström and Zetterström 1954), and its staining characters with haematoxylin ranging from deep blue suggestive of calcification (Pines and Lederer 1947, Zawisch 1947) to lightly staining osteoid tissue in amounts sufficient to simulate rickets (Kramer and Halpert 1939; Kramer, Yuska and Steiner 1939; Cohen 1951; Folliis 1952).

Within and at the surface of bone, wide "cementing" lines staining deeply with haematoxylin occur between layers of bone in which remodelling has been negligible (Zawisch 1947). Microradiographs show areas of high and low bone density with exaggerated thick radio-opaque "cementing" lines both on the surface and concentrically around immature Haversian systems. Occasionally intense bone resorption has been observed (Engfeldt et al. 1954; Engfeldt, Karlberg and Zetterström 1955). Cellularity varies greatly: osteoblasts and osteoclasts may be within normal limits (Enticknap 1954), osteoblasts at the trabecular margins may be merely small spindle cells (Zawisch 1947), or there may be areas of bone formation and active resorption (Engfeldt et al. 1954).

Changes are most obvious in, and usually confined to, the bones but may be accompanied by a generalised calcinosis with calcification of the main arteries of the limbs, ligaments, larger tendons, kidneys, trachea, stomach and other soft tissues (Fairbank 1948).

Biochemical results disagree, probably because observations are made at different stages of the disease: the serum calcium level is usually within normal limits (Ellis 1934), but occasionally raised levels have been found (Platt, Erhard and Araj 1956). Serum phosphorus is often low and the Ca×P product low enough to suggest rickets (Pincus, Gittleman and Kramer 1947; Cassidy, Allman and Keefe 1948; Pines and Lederer 1947), as does the finding that bones contain more carbonate than the normal bones of infants of the same age (Kramer et al. 1939).

By contrast, an adult failed to show abnormality of calcium salts or of hydroxyproline relative to bone salt (Enticknap 1954). Patients with osteopetrosis associated with rickets may be extremely resistant to vitamin D therapy and respond only slowly to massive doses (Kramer and Halpert 1939, Pincus et al. 1947).

The obscure nature of the changes has stimulated many hypotheses. Suggestions such as a faulty differentiation and development of the mesenchyme (McCune and Bradley 1934) or a diseased vascular and osseomedullary anlage (Pines and Lederer 1947) do not deserve or require special attention. As to the mechanisms underlying the changes, many writers consider that the phenomena are the result of a reduction in osteoclastic activity during bone growth (Weinmann and Sicher 1955). Snapper (1957), in a recent discussion of the pathogenesis, has pointed out that, since typical areas of bone resorption may be demonstrated (Engfeldt, Karlberg and Zetterström 1955), inhibition of bone resorption cannot be the sole cause of the dense bone.

From the etiological viewpoint, Ellis (1934) suggested that intermittent hyperparathyroidism might be responsible. In 1932 Selye remarked on the bone sclerosis in the rat following administration of parathormone, but similar changes have since been reported in the
rat with oestrogens (Day and Folli 1941), vitamin D (Shelling and Asher 1932) and
cortisone (Folli 1951). One observer has given appropriate attention to the outstanding
feature of the disease; Zawisch (1947) considered that the bands of greater or less bone density
could occur in two ways: ‘‘(a) by periodic remission and recursences of the pathologic
deposition of bone and (b) by periodic recurrences of widespread resorption.’’ Although all
the contingent details may not be completely acceptable, this is a noteworthy adumbration of
present experimental observations.

In summary, one consistent feature of the bone changes in osteopetrosis is the alternation
of bands of dense and less dense bone, particularly in the metaphysial region of growing
long bones. Such variations in structure (and presumably in tissue activity) are associated,
depending on the phase and the area of the bone studies, with histological changes of deposition
and resorption. The analysis of the bone changes by Zawisch (1947), postulating that excessive
deposition would be followed by a swing in the opposite direction and thus excessive bone
resorption, are in accord with our present understanding of withdrawal phenomena (Storey
1958). In view of the differences found between examples of the condition, and despite the
amount to which biological variation may influence the range of appearances found in any
condition, it is important to consider that osteopetrosis may not necessarily be a single disease
with a single precise etiology. The presence of rickets or rickets-like lesions in these patients,
particularly in view of the remarkable resistance to vitamin D therapy, indicates that more
information is necessary on the fundamental influences of vitamin D.

EXPERIMENTAL PROCEDURES

One hundred and fifty rats of both sexes (Sprague-Dawley and Wistar) weighing 70–150
grammes were used. They were fed on a diet of Barastoc pellets—a local proprietary food
containing 1·6–1·8 per cent calcium and 0·9–1·1 per cent phosphorus—with fresh greens,
vegetables and water as required.

Calciferol dissolved in arachis oil (1 milligram containing 40,000 I.U. in 1 cubic centimetre)
was administered by stomach tube, the dose being 10 milligrams/kilogram/day. Rats were
killed at intervals during the experiments, and their bones were removed and examined
macroscopically before being fixed in a solution of neutral formol saline. Decalcification was
carried out in 5 per cent nitric acid, or as described by Menzies and Mills (1957). After washing,
bones were embedded in paraffin wax, sections cut at 5–7 μ and stained with either Ehrlich’s
or Weigert’s haematoxylin and eosin, periodic acid-Schiff, toluidine blue, Alcian blue, silver
impregnation and Schmorl’s canaliculi stain.

Experiments in two main groups were: first, to determine the effect of administration of
calciferol and its withdrawal; and secondly, to determine the effect of intermittent
administration of calciferol; in the text the number of days of administration is signified by
(−) and the duration of withdrawal by (−).

The individual experiments were as follows:
Administration and withdrawal of calciferol—control animals (10); continuous administration
of calciferol for sixteen days (10); withdrawal of calciferol after one to four days’ administration
(10).
Intermittent administration of calciferol—control animals (10); calciferol +4 −12 days, the
cycle being repeated three times (10); calciferol +1 −12 and +1 −21, the cycle being repeated
in one series five, in a second seven times: these cycles are only two of several investigated (100).

RESULTS

ADMINISTRATION AND WITHDRAWAL OF CALCIFEROL

Control animals—Control animals at all stages showed normal growing bones (Fig. 1).
Continuous administration of calciferol—In four days the epiphysial cartilage plate was
slightly narrowed and metaphysial trabeculae were diminished by increased resorption (Fig. 2).
With continued administration the cartilage became narrower, but areas of bone resorption were less obvious as more osteoid material formed at the bone margins, particularly at sites of normal membrane bone growth such as the periosteal surfaces of the shafts of long bones and the bony sutures of the skull. After sixteen days a mixed picture of resorption of metaphysial bone near the cartilage and widened osteoid seams on adjoining bone trabeculae was obvious, and some haematoxylin-staining granules were now present at the junction of bone and osteoid material. By this time animals had lost considerable weight and were weak and listless; at necropsy metastatic calcification was present in the viscera and aorta.

Withdrawal of calciferol—In the first experiment calciferol was administered for four days and then discontinued. At this time increased resorption was obvious, but four days after withdrawal the margins of epiphysial and metaphysial trabeculae were nearly covered by a thin layer of osteoid material and signs of bone resorption were reduced. After eight days this osteoid layer was wider and ubiquitous, being least obvious at the endosteal and periosteal surfaces near the middle of the shafts of long bones. At this time bone resorption was almost completely inhibited and the epiphysial cartilage still remained narrowed.

Twelve days after withdrawal the growth of epiphysial cartilage was resumed. The osteoid layer reached its greatest width and bone surfaces were lined by osteoblasts (Fig. 3). The junction of the original bone and osteoid material was marked by a faint to dense blue staining line which was irregularly scalloped and showed the extent of previous resorption. Such lines indicated the site where either resorption or deposition of bone had ceased and had been followed by tissue activity in the opposite direction. This resulted in the local formation, at this site, of a deeply staining material which has received various definitions. Since the
essential functional alteration is a change in the direction of tissue activity the term "reversal" has been employed. The osteoid material failed to stain intensely with haematoxylin and periodic acid-Schiff and was irregularly granular with toluidine blue; the osteocytes were larger than those of normal bone and their canaliculi did not stain with Schmorl's stain. Silver impregnation revealed the loose fibrillar woven appearance of the matrix.

After twenty-one days, macroscopic examination showed a dense band of bone in the metaphysial area some distance from the epiphysial cartilage; this was opaque and was cut with difficulty. Histologically the epiphysial cartilage was wider, and a newly formed metaphysis consisted of short trabeculae (Fig. 4). Separated from these trabeculae by a layer of marrow cavity was a dense plate of bone consisting of a mass of osteoid material enclosing cartilaginous remnants of metaphysial trabeculae. Osteoid material now stained faintly with haematoxylin and periodic acid-Schiff; a few margins were irregular and scalloped while the remainder were lined by osteoblasts. Remnants of this dense band were still present after three months, vascular channels and scalloped margins indicating the presence of bone resorption.

In a second experiment after a single dose of calciferol the withdrawal period showed large amounts of osteoid material, but, in contrast with the previous experiment, the junction between the original bone and newly formed osteoid material was less scalloped and more regular.

**INTERMITTENT ADMINISTRATION OF CALCIFEROL**

**Control animals** showed normal bones at all stages of the experiment.

**Calciferol administration** +4 —12, +4 —12, +4 —12. **Macroscopic changes**—After the first cycle (+4 —12) of calciferol administration, bones were easy to cut and the epiphysial and

---

**Figure 3**—Photomicrograph of metaphysial trabeculae from the tibia of a rat twelve days after withdrawal of calciferol. Wide osteoid seams cover metaphysial bone margins. (Periodic acid-Schiff haematoxylin, × 90.) **Figure 4**—Photomicrograph of tibia of a rat twenty-one days after withdrawal of calciferol. A wide transverse band is seen some distance from the metaphysis and consists of cartilaginous cores covered with osteoid seams partly stained with periodic acid-Schiff. (Periodic acid-Schiff haematoxylin, × 10.)
widened metaphysial regions of the tibia and femur consisted of white opaque bone; similar changes were present at the sutures of the skull. During the next period of vitamin D administration bones were redder and a dark line separated dense metaphysis from epiphysial cartilage, but during withdrawal the bone again appeared opaque and white. After three cycles bones were extremely difficult to cut; even the relatively thin cranial vault was so hard that scalpel blades broke. Separation into layers was not so obvious at this stage in the long bones, and they appeared to be uniformly dense at the epiphysis and metaphysis.

Calcification was found in the aorta, stomach and kidney, and the experiment was stopped because of the weakened condition of the remaining animals.

**Microscopic changes**—The sequence of events will be described in both membrane and cartilaginous bone—exemplified by appositional growth of the skull and cartilaginous growth of the upper end of the tibia. After this, individual bone changes will be described.

![Photomicrograph of section of normal cranial bone. (Ehrlich's haematoxylin and eosin, × 225.)](image)

1) **Membrane bone formation**—At the end of the first cycle (+4 – 12) a wide layer of osteoid material outlined bone margins (Figs. 5 and 6). After a further four days of calciferol administration (+4 – 12, +4) scattered areas of resorption were present and a denser layer of haematoxylin-staining granules appeared at the junction of the original bone and the newly formed osteoid substance. Osteocytes near this layer in the osteoid were now enclosed by a fine line of haematoxylin-staining material, and similar material marked their canaliculi. By the end of the second withdrawal phase (+4 – 12, +4 – 12) a second layer of osteoid material had formed on the surface of the first, separated from it by a granular layer faintly stained with haematoxylin (Fig. 7). The first osteoid layer by this time was infiltrated by a fine granular haematoxylin-staining material. When vitamin D was given for a third time some resorption of already calcified bone occurred and concurrently the second osteoid layer became infiltrated with haematoxylin-staining granules: the initial deposit formed a denser line at the junction of the first and second osteoid layers (Fig. 8). At this stage the first osteoid
FIG. 6
Photomicrograph of section of cranial bone after one cycle of calciferol administration (X 4 — 12). A new wide osteoid seam of woven bone has formed separated from the original lamellar bone by an irregular lightly staining reversal line. (Ehrlich's haematoxylin and eosin, X 225.)

FIG. 7
Photomicrograph of section of cranial bone after two cycles of calciferol administration (X 4 — 12, X 4 — 12). The osteoid material seam formed during the first cycle is now infiltrated deeply with haematoxylin-stained granules, particularly the reversal line adjoining the original lamellar bone. In contrast the second osteoid material seam fails to stain with haematoxylin. (Ehrlich's haematoxylin and eosin, X 225.)
Photomicrograph of section of cranial bone after three cycles of calciferol administration (1-4 -12, 4-12, 4 -12). The first osteoid material seam now appears less granular, while the second seam and reversal line are now infiltrated with densely staining material. Note that a third distinct reversal line has failed to form. (Ehrlich's haematoxylin and eosin, × 225.)

Photomicrograph of section of cranial bone after three cycles of calciferol administration (1-4 -12). This shows three layers of bone: first (0) the original bone in which irregular remodelling has obliterated most of the marrow spaces and distorted the normal structure; the first osteoid material seam (1) now calcified with most bone margins marked by reversal lines; the second osteoid seam (2) consists of a mass of osteoid substance partly covered by haematoxylin-stained granules in which quite large vascular spaces are present. (Ehrlich's haematoxylin and eosin, × 125.)
substance layer had lost its granular appearance and become more mature, being distinguishable from lamellar bone only by the enlarged osteocytes and woven matrix. Significant modelling of this bone layer did not occur, but new small Haversian systems indicated limited resorption; this was followed by further deposition and new reversal lines at bone margins. At the end of the third cycle bone was added to the existing surfaces as outlined above, but no distinct reversal line could be detected at surfaces that were not calcified; marrow spaces became filled with layers of bone from the endosteal surface. Eventually membrane bones became extremely sclerotic, with almost complete obliteration of the marrow cavity or vascular channels (Fig. 9). When sections were stained with periodic acid-Schiff or haematoxylin the outermost layer of osteoid material failed to stain.

2) Cartilage bone growth—At the end of the first cycle of calciferol administration (+4 – 12) a wide layer of osteoid material was laid down at metaphysical trabecular margins and adjoining the epiphysial cartilage (Fig. 10); instead of normal remodelling of bone a subperiosteal layer of osteoid substance formed laterally, distorting the metaphysis. After the next period of calciferol treatment (+4 – 12, +4) resorption of bone took place near the epiphysial cartilage, where remnants of the new metaphysis were present. During the next withdrawal period (+4 – 12, +4 – 12) osteoid material was again laid down on these trabeculae to form a second transverse band of dense bone with enclosed cartilage cores.

A layer of osteoid substance appeared between the first and second transverse band; this contained many small Haversian systems (Fig. 11). The first formed osteoid layer now stained deeply with haematoxylin in contrast with that layer now contiguous with the epiphysial cartilage. The first band of dense bone showed some remodelling, with removal
of both cartilage and osteoid material and its fenestration by small Haversian systems; concomitantly a new layer of periosteal osteoid material formed so that the metaphysial outline became club-shaped (Fig. 12).

With the third cycle (+4 −12, +4 −12, +4 −12) osteoid material was added to trabecular surfaces but no dense transverse band formed because no cartilage growth had taken place during the withdrawal period. In the epiphysis chondrocytes decreased in number and intercellular substance increased in some areas. By this time the epiphysial bone consisted of a solid block, bone obliterating most of the marrow cavity. In contrast with this the central part of the shaft of the bone was unaffected.

3) Individual bone changes—Individual bones and different areas of the same bones showed considerable variation. For example, the metaphysial bone was wider and denser at the upper than at the lower end of the tibia; the lower end of the femur showed less dense bone than the adjoining part of the tibia whereas the head and neck of the femur were grossly changed with little alteration to the diaphysis.

In sections of the skull the cranial vault was almost a solid sheet of bone, with marrow spaces obliterated by layers of osteoid material; sutures became narrow or even obliterated (Fig. 13). Little periosteal osteoid material formed on the cranial vault in contrast with the base of the skull, where layers were laid down on both the internal and the external surfaces to form a large block of bone (Fig. 14).

In addition to sclerosis of bone, calcification of ligaments was most obvious in the vertebral column (Fig. 15), and the vertebral epiphysial plates were extremely sensitive to this cyclical stimulation.
In the jaws the most obvious change consisted of partial ankylosis of the permanent molar teeth due to encroachment on the periodontal membrane by osteoid material formed on both lamina dura and cementum. The continuously growing incisor teeth were not ankylosed. **Calciferol administration** (+1 -12 and +1 -21). In this series of experiments various types of cycles were investigated in attempts to prolong the effect of vitamin D without killing the animals. The duration of calciferol administration was first reduced, then the withdrawal period was lengthened. Two such cycles are described.

**Macroscopic changes**—Bone changes in the metaphysial region of long bones were extremely variable in this series. They ranged from the formation of uniformly dense metaphysical bone and dense transverse bands parallel to the epiphysial cartilage to remnants of dense trabeculae extending into the marrow cavity (Fig. 16). In all cases the number of transverse bands of bone fell short of the number of cycles of calciferol administration.

**Microscopic changes**—In addition to the three types of bone change observed macroscopically, gradations intermediate in structure between these, as well as combinations of the three, might exist in the same or different bones of the same animal. The three main types of bone change were:

1) **Dense sclerotic bone**—The epiphysis consists of a dense mass of bone (containing some irregularly arranged Haversian systems) which obliterated most of the marrow cavity (Fig. 17). The epiphysial cartilage was extremely narrow and in some areas bands of largely cell-free cartilaginous matrix extended down through the metaphysis to form longitudinal striations (Fig. 18). This cartilaginous matrix contained no fibres staining with either periodic acid-Schiff or silver in contrast with normal cartilage matrix; in addition, numerous large globules, which
Fig. 16
Photographs of bones from control and two calciferol treated rats showing variation in bone changes (7 cycles, -1 -21). Femora are shown above and tibiae below. ( -6.) 1) Control rat. 2) Calciferol administration. Dense bone present in the epiphysis and metaphysis of the femur, while the tibia shows three transverse bands of dense bone. 3) Calciferol administration. Dense trabeculae present in metaphysis of femur; in the tibia they are reduced in number and irregularly arranged. In both bones a dark line is present next to the epiphyseal cartilage where new bone has not yet formed following the last withdrawal period.
Figure 17—Photomicrograph of femur after five cycles (±1–12) of calciferol administration. Extreme sclerosis of bone is present both in metaphysis and epiphysis. Metaphysial trabeculae instead of being resorbed are smoothly rounded. (Weigert's haematoxylin and eosin, ×10.) Figure 18—Photomicrograph of metaphysial area from Figure 17 showing darkly staining accumulations of abnormal cartilage matrix within the dense bone. (Weigert's haematoxylin and eosin, ×90.)

Photomicrograph of metaphysial area cut in cross-section showing the distribution of abnormal cartilage matrix within the bone after five cycles (±1–12) of calciferol administration. This matrix stains irregularly with Alcian blue and is continuous in places with thin lines of normal cartilage remnants. (Alcian blue and haematoxylin, ×200.) (Photographed on Panchromatic film with a red filter.)
Photomicrograph of metaphysial area seen in Figure 17. Considerable remodelling has occurred although deeply staining cartilage remnants are still present. Considerable variation exists in size and staining of Haversian systems. (Ehrlich's haematoxylin and eosin, × 200.)

Figure 21—Photomicrograph of metaphysial area from vertebra after five cycles (+1 -12) showing parallel longitudinal striations of abnormal cartilage matrix present in dense bone. (Weigert's haematoxylin and eosin, × 160.)

Figure 22—Photomicrograph of lower end of femur after five cycles of calciferol administration (+1 -12). Remains of four transverse bands still persist in the marrow cavity. (Ehrlich's haematoxylin and eosin, × 10.)
FIG. 23
Photomicrograph of part of the shaft of the femur after five cycles of calciferol administration (-1 -12). Remodeling of calcified cartilage has not yet progressed to any extent. (Weigert's haematoxylin and eosin, ×125.)

FIG. 24
Figure 24—Photomicrograph of tibia after seven cycles of calciferol administration (-1 -21). The epiphysial bone is dense but only two thin transverse bands and a number of longitudinal trabeculae are seen in the metaphysial region. (Weigert's haematoxylin and eosin, ×10.)

FIG. 25
Figure 25—Photomicrograph of shaft near the lower end of the tibia after seven cycles of calciferol administration (-1 -21). Trabeculae persist and increase in size due to accretion of thin layers of bone instead of being removed during remodelling. The shaft of the bone is on the left-hand side of the photograph. (Weigert's haematoxylin and eosin, ×60.)
in places stained strongly with haematoxylin, Alcian blue and periodic acid-Schiff, were suggestive of calcification (Fig. 19).

Metaphyseal bone showed considerable remodelling, with removal of cartilage and osteoid material and development of Haversian systems (Fig. 20). Trabeculae instead of being removed persisted and increased in size to form large rounded interlocked structures which in cross-section showed accretion of concentric layers. Haematoxylin stained the inner more deeply than the outer layers even though osteoid material might not be particularly prominent at the bone margins.

Despite extreme sclerosis of epiphysial and metaphysial bone the diaphysis was largely unaffected. In the vertebral column abnormal longitudinal striations of cartilage matrix were most obvious (Fig. 21).

2) Transverse bands of sclerotic bone—Wide metaphysial trabeculae, consisting of unresorbed cores of calcified cartilage surrounded by layers of bone and osteoid material, formed transverse bands lying parallel to the epiphysial cartilage and extended across the marrow cavity (Figs. 22 and 23). The space between the layers consisted of marrow cavity and a few remnants of bone trabeculae. Longitudinal striations of relatively acellular cartilage matrix persisted within the dense bone, and in some cases were present at the metaphysis adjoining the epiphysial cartilage. During remodelling these striations were resorbed concomitantly with the surrounding bone.

3) Remnants of sclerotic trabeculae—In some bones minimal sclerotic changes were present and remnants of trabeculae extended down into, or across, the marrow cavity (Fig. 24). Some of these enclosed cores of unresorbed cartilage; others were smoothly rounded due to peripheral addition of layers of bone. These changes were more prominent at slowly growing sites of endochondral growth such as the lower end of the tibia (Fig. 25).

DISCUSSION

The continuous administration of massive doses of vitamin D was associated with inhibition of endochondral growth and initially with resorption at osseous surfaces, but after this a dense mass of osteoid material of unusual structure formed. This has been described before, the condition being termed "hypervitaminosis D rickets" (Ham and Lewis 1934). Such reversal of the usual and initial change was well seen during continuous administration of hormone in the rat (but not the rabbit), especially with parathormone (Selye 1932, Shelling and Asher 1932) and cortisone (Follis 1951, Storey 1960). After a short period of administration followed by withdrawal of vitamin D a more obvious reversal effect resulted in excessive production of new osteoid material which over-compensated for that originally lost. This mechanism was also well demonstrated in rabbits after withdrawal of cortisone (Storey 1958).

Osteoid material, formed after brief administration of vitamin D, did not calcify for some time, but eventually haematoxylin-staining material indicating calcification appeared, forming a reversal line, and infiltrated through the matrix in a patchy manner. It has been suggested that this osteoid material was abnormal in having lost its calcifying capacity (Follis 1956); however, as shown by Follis, not only will this rachitic tissue calcify when placed in the animal's serum in vitro (and this applies also to calcium salt solutions), but if, at a later date, large doses of vitamin D are given the matrix calcifies in vivo. The bone changes are thus dependent on as yet unelucidated systemic disturbances. Incidentally, it was evident that vitamin D not only influenced calcium metabolism but, either directly or indirectly, affected the organic component and controlled the rate of formation of osteoid material. This emphasises that the two constituents of bone tissue—inorganic and organic—may be influenced independently of each other and, furthermore, by the same agent.

If, after an interval of some days, vitamin D was given again calcification of osteoid material formed during the first withdrawal period was accelerated. The first sign, near the
junction of original bone and new osteoid material, was a wide dense granular haematoxylin-staining line. The rest of the osteoid material calcified in the next few days. Bailie and Irving (1955) showed that the healing of rickets in rats also began at the junction of calcified bone and osteoid material, although in their experiments no such thick line was seen. These thick lines indicate an abnormality of the "local factor" (Sobel 1955), for periodic acid-Schiff staining showed an absence of this mucopolysaccharide in uncalcified osteoid substance and the appearance of excessive amounts during calcification. Similarly dense reversal lines have been induced by intermittent injection of cortisone in the rabbit (Storey 1958), but the present experiments showed more clearly the discrepancy between matrix formation and calcification.

With several short administrations of vitamin D at intervals, cycles of bone resorption were followed by waves of excessive formation of osteoid material: bone formation so exceeded removal that eventually after calcification bones became thicker, denser and harder. The method of accretion of bone ranged from simple addition of layers as flat plates, as seen in the skull, to complicated transverse bands in the metaphyseal area in which unabsorbed cartilage was retained. Though the osteoid material laid down at earlier stages became calcified, and indeed may be hypercalcified as judged by the difficulty in cutting it with a scalpel, the last formed layer remained uncalcified. The degree of change varied throughout the skeleton, but in general only growing areas were affected. The cranial vault became dense but not much thicker whereas the base of the skull became extremely dense and thick; the metaphysis and epiphysis of long bones became extremely sclerotic whereas the middle of the diaphysis was unaffected. Different animals showed considerable variations, which probably reflect differences in rates of remodelling of bone; these were shown most clearly in the metaphyseal area, where endochondral growth may become intermittent. Here bone may be extremely and uniformly dense, it may have sharply defined sclerotic bands separated by normal bone or marrow cavity, or it may show either minimal sclerosis with thick dense trabeculae persisting in the marrow cavity or little sign of any cyclical activity except for layers of bone added to existing trabeculae.

It was clear that when the period of vitamin D withdrawal was insufficient for normal remodelling processes to occur, uniformly dense bone formed; when normal remodelling took place between sclerotic episodes normal bone or marrow cavity separated the dense bone into transverse bands; finally as the period of remodelling increased little sign of dense bone remained.

In addition to these perversions of the remodelling process the epiphyseal cartilage could be altered in such a way that parallel longitudinal striations were produced in the bone. These consisted of wide lines of cartilage matrix of a fibrillar consistency which arose from defects in the continuity of the epiphyseal plate and, as growth continued, were incorporated into the bone substance. It should be noted that longitudinal striations were a feature of some cases of osteopetrosis, but their nature was not elucidated. Defects of this nature in the epiphyseal cartilage have been observed previously in the spines of normal men (Beadle 1931) and in old rats (Dawson 1929). In our experiments these defects occurred at a much younger age and could merely represent premature ageing of the cartilage plate associated with continued interruptions of cellular activity.

When these bone changes are compared with those of osteopetrosis, both may show clubbing and sclerosis of the long bones with parallel transverse bands and longitudinal striations at the metaphysis, parallel seams at sites of appositional growth, areas of extremely high and of extremely low density and, rather than lamellar bone, abnormal woven matrix with histological evidence of wide seams of osteoid material. The extra-osseous calcification of ligaments, blood vessels and viscera in the rat was unlike that seen in osteopetrosis; recent work (Gillman and Gilbert 1956, Selye 1958) may point the way in which hormones may modify the effect of large doses of vitamin D, so that the bone changes are produced independently of raised serum calcium levels and metastatic calcification.
Ellis (1934), from radiographic studies, suggested that osteopetrosis might be associated with intermittent parathyroid activity, and similarly microscopic studies showing wide dense reversal lines also directed attention to the probability that the process was not continuous but intermittent (Zawisch 1947). Zawisch (1947) suggested an acceleration of the "microrhythm" of bone change, either resorption or deposition being associated with over-compensation in the opposite direction. This was duplicated with vitamin D administration in the present study in that compensatory production of bone far outstripped the initial resorption. Nevertheless some points in her paper are opposed to our observations: first, dense reversal lines do not inhibit bone resorption, as is demonstrated by their disappearance in lacunae during remodelling (Storey 1958); furthermore, although not present in Zawisch's case, osteoid material (rickets-like bone) is found in some recorded cases. Differences of histological (as of radiological) structure will necessarily be found in a cyclical and changing process depending on the stage at which the condition is examined.

The association of hypercalcification of bone and uncalcified osteoid material (rickets) in the one bone at the same time was noteworthy. Although at first sight rickets appeared an unlikely prerequisite for production of excessive amounts of hard bone, consideration of basic processes rather than diseases resolved the difficulties. Loosely woven matrix would be expected to allow deposition of a greater amount of bone salt relative to organic material than lamellar matrix. This is consistent with the physical behaviour of bone. The present experiments emphasised the extreme difficulty of interpretation of results of biochemical analysis of bone where wide variation in structure occurred at the microscopic level.

Malocclusion of the jaw was associated with the continuous growth processes of the rat; even old animals retained their epiphysial cartilages and continued to grow slowly. Interference with growth, as by cortisone, produced malocclusion in suckling rabbits, the deformity occurring in the recovery period when the times of renewal of growth of the skull and mandible were different. Similar mechanisms are probably responsible for malocclusion in the present study; the skull which became sclerotic, with ankylosis of sutures, failed to grow in the withdrawal period whereas the mandible, showing less sclerosis at the condyle, continued to grow forwards so that the lower teeth eventually protruded in front of the upper ones. Arrest of growth, with recovery of different bones at different rates and times, was important in the etiology of malocclusion and points the way to a method of study of other abnormalities.

At this stage the relation of vitamin D activity to osteopetrosis must be speculative. Accumulated evidence points to a range of sensitivity to this vitamin, from almost complete "resistance" to "hypersensitivity" with signs extending from rickets to sclerosis of bone (Fanconi 1956). It is perhaps relevant to ask here whether osteopetrosis can be placed at one end of the range.

It should be emphasised that these experimental results do not indicate the etiology of naturally occurring diseases such as osteopetrosis, but rather that they demonstrate the type of fundamental change responsible for them. This is some form of cyclical stimulus or cyclical interruption of a continuous stimulus, the relative times respectively of resorption and deposition determining the exact form and site and degree of the bony change encountered in a given case.

**SUMMARY AND CONCLUSIONS**

When large daily doses of vitamin D were administered to rats endochondral growth was inhibited and bone resorption occurred; later in the process uncalcified matrix (osteoid) like that seen in rickets formed on trabecular margins. When vitamin D was given only for a short period and then discontinued, little resorption of bone was seen during the withdrawal period and wide seams of osteoid material appeared which eventually calcified in an irregular manner. When normal endochondral growth was resumed a wide transverse band of dense bone with enclosed cartilaginous cores was left in the marrow cavity. If, after
a few days, a second large dose of the vitamin was given resorption again occurred and calcification of osteoid material was accelerated, the first microscopic sign being a dense, wide, granular, deeply staining line at the junction of the bone and new osteoid. After a second withdrawal period a second layer of osteoid formed; eventually another transverse band appeared in the metaphysis. If this hypervitaminosis D cycle (+4 – 12) was continued rats continued to form new bone with relatively little remodelling, so that after three such cycles bones became dense and hard.

Histological study showed that little marrow cavity remained in either skull, vertebrae or epiphyses and a dense mass of bone enclosing cartilage cores filled the metaphysial part of the long bones. In addition, ankylosis of teeth, calcification of spinal ligaments and widespread metastatic calcification were present.

When hypervitaminosis D cycles (+1 – 12, +1 – 21) were adjusted to produce minimal resorptive changes a wide range of bone change was observed. This varied from uniform dense metaphysial bone containing abnormal cartilage matrix arranged in longitudinal striations, dense transverse bands parallel to the epiphysial cartilage, to remnants of dense trabeculae extending into the marrow cavity.

Bone changes in osteopetrosis structurally closely resembled the induced bone changes in the rat. It is concluded that an important mechanism in the production of osteopetrosis is an accentuated rhythm of bone change like that shown experimentally to be produced in these animals. It is emphasised that these changes are but part of a range of bone disorders associated with abnormalities of cycles of resorption and deposition of bone, the type of change differing with the nature of the cycles.

This work was carried out under a grant from the National Health and Medical Research Council of Australia.

REFERENCES


CASSIDY, W. J., ALLMAN, F. C., and KEEFE, G. J. (1948): Osteopetrosis. Archives of Internal Medicine, 82, 140.


ELLIS, R. W. B. (1934): Osteopetrosis (Marble Bones; Albers-Schönberg's Disease; Osteosclerosis Fragilis Generalisata; Congenital Osteosclerosis). Proceedings of the Royal Society of Medicine (Section for the Study of Disease in Children), 27, 1,563.


HAM, A. W., and LEWIS, M. D. (1934): Hypervitaminosis D Rickets, the Action of Vitamin D. British Journal of Experimental Pathology, 15, 228.


