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Osteopetrosis Fetalis

Report on a Case, with Special Reference to Ultrastructure

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Summary. The clinical and pathological findings concerning the skeletal abnormalities in a case of osteopetrosis fetalis have been reported. The principal data can be summarized as follows.

The areas of endochondral ossification have a rickety appearance because of excessive number of hypertrophic and degenerate chondrocytes. These cells are highly vacuolated and the vacuoles, which are of mitochondrial origin, contain beaded filaments which are exocytosed and become part of the matrix. The calcification process is delayed, probably in consequence of a reduced number of matrix vesicles. Abnormal collagen fibrils are sometimes present in the cartilage. The osteoclasts have a very low reabsorbing activity and appear structurally abnormal.

The combined effect of all these abnormalities leads to excessive development of osteocartilaginous trabeculae in marrow spaces. These trabeculae have a Ca/P ratio of 1.79 and their mineral substance appears qualitatively normal under the electron microscope.

Key words: Osteopetrosis — Bone pathology — Bone ultrastructure.

Osteopetrosis is a heritable disorder of the skeleton, chiefly characterized by abnormal bone density and brittleness, and obliteration of the medullary spaces by calcified osteo-cartilaginous trabeculae (Jaffe, 1972). The pathogenesis of the disease remains uncertain and it is still dubious if there is overproduction of bone tissue or reduced resorption, and if the abnormal structure of bones is due to a defective activity of the osteoclasts or to changes of the structure and composition of the calcified matrix. The uncertainty is increased by the fact that osteopetrosis can occur in at least two forms, one leading to early death (osteopetrosis fetalis, malignant form), the other (osteopetrosis tarda) not incompatible with longevity (McKusik, 1972).

Electron microscope investigations of osteopetrotic bone are exceedingly rare. Höhling and Czitober (1971), studying bone biopsies from two adult patients with osteopetrosis tarda, reported the presence of areas containing needle-shaped and "chain like" calcium phosphate deposists and lacking collagen fibrils.

Recently, we have had the opportunity of studying a costo-chondral biopsy from a male baby with osteopetrosis fetalis. We feel it useful to report the results we have obtained, because for the first time the histological findings can be compared with those obtained with histochemical, biochemical and electron microscope techniques.

Case Report

F. F., 5-month-old male baby, first child of a healthy woman. Normal delivery after a 40-weeks uncomplicated pregnancy. Weight at birth 3450 g. Normal growth and development during the first two months of life. Successively, recurrent periods of vomit and diarrhoea.

Admitted to the Pediatric Clinic of the University of Padua, he appeared pallid and was severely dystrophyc. His skeleton was not deformed, but the x-ray examination (Fig. 1) showed highly increased density of the cortical bone of all the skeletal segments, reduction or complete disappearance of marrow cavities, and distorted and irregular transverse metaphyseal lines. The liver and the spleen were prominent. Moreover, the patient had severe anemia and thrombocytopenia with haemoglobin value of 7 g/100 ml and presence in the blood of erythroblasts and metamyelocytes (40000 mmc).

The patient was initially treated with vitamin D_3 associated to hypocalcemic diet. This therapy produced a decrease of the calcemia (from 8.0 to 5.8 mg/100 ml) without signs of tetany, reduction of bone density, and improvement of rickety appearance of the metaphyses. Moreover, a splenectomy was performed at 4 mounts of age with the hope of improving the haemopoietic disorder. In spite of this intervention and of repeated blood transfusions, the patient died at 5 months of age with severe anemia and debilitation.

Material and Methods

A small specimen of a costo-chondral synchondrosis was removed during splenectomy. It was immediately soaked in 4% paraformaldehyde buffered at pH 7.2 with cacodylate buffer, and was divided lengthwise. One part was used for histological and histochemical investigations. It was decalcified with formic acid and embedded in paraffin. The other part was reduced to small fragments which were left in paraformaldehyde for two hours, post-fixed with 1% osmium tetroxide for 1 hour, and embedded in Araldite after acetone dehydration.

A segment of the femurs was taken during the autopsy. Part of it was fixed with paraformaldehyde, decalcified with formic acid and embedded in paraffin. The other part was used for biochemical analysis. Sections from paraffin embedded specimens were stained with: (a) hematoxylin and eosin for routine examination; (b) Mallory's method for collagen; (c) periodic acid-Schiff (PAS) for glycoproteins; (d) 1% alcian blue, pH 1.8, and colloidal iron (Mowry, 1958) for acid proteoglycans; (e) von Kossa method for calcium phosphate. Semithin sections from Araldite embedded specimens were examined after staining with azure II and methylene blue. Ultrathin sections for electron microscopy were examined unstained and after staining with uranyl acetate and lead citrate.

Parts of heart, liver and kidneys were examined for their water, calcium and phosphorus contents together with the remaining part of the femur. Calcium and phosphorus were analysed by atomic absorption spectrometry (Parson *et al.*, 1970), each sample having been digested in perchloric acid (70%) —H_2O_2 at 120° C and the acid evaporated in a sand bath. Water content was examined by storing the tissues in a desiccator over P_2O_5 at 60° C until a constant weight was achieved.

Results

As shown in Fig. 2, the *microscopic morphology* of the long bones was completely similar to that already reported in subjects with osteopetrosis: the marrow spaces were occupied by irregular trabeculae formed by an inner calcified cartilaginous core surrounded by bone tissue. Under the electron microscope, the cartilaginous core appeared more electron-dense and compact than the peripheral, osseous part (Fig. 3). However, as a whole, the fine structure of these osseous and cartilaginous trabeculae was similar to that of the trabeculae found in the ossification zone of normal epiphyseal plates.

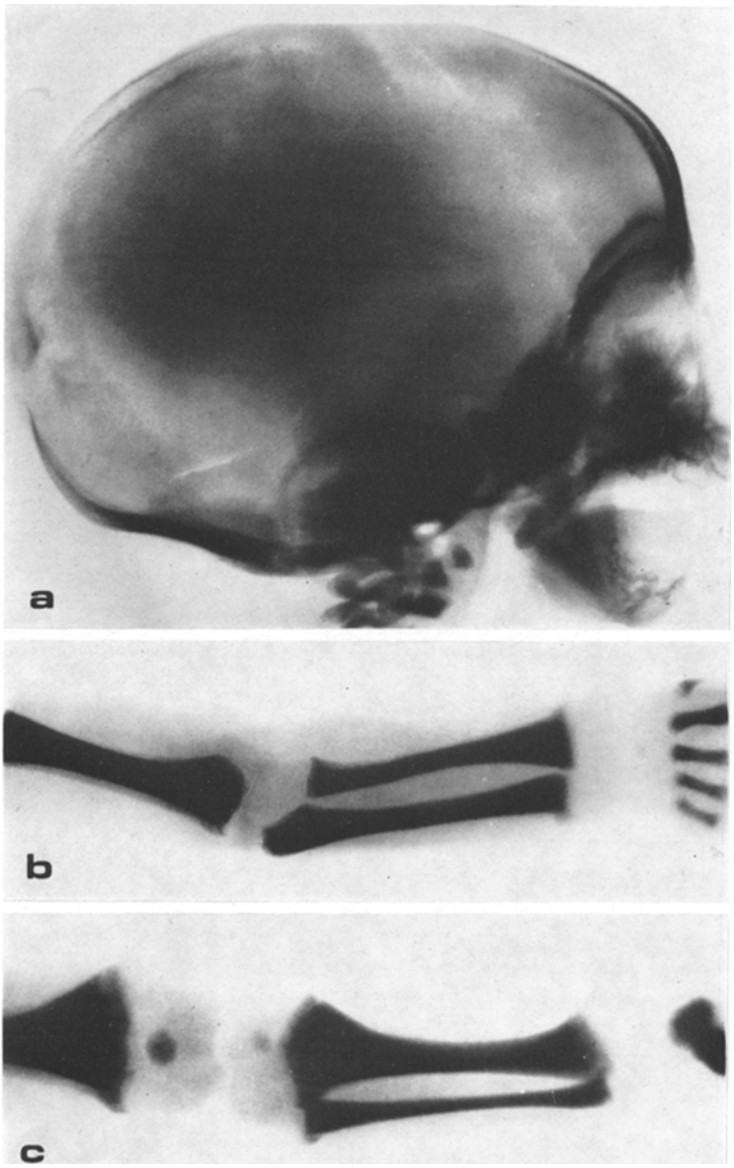
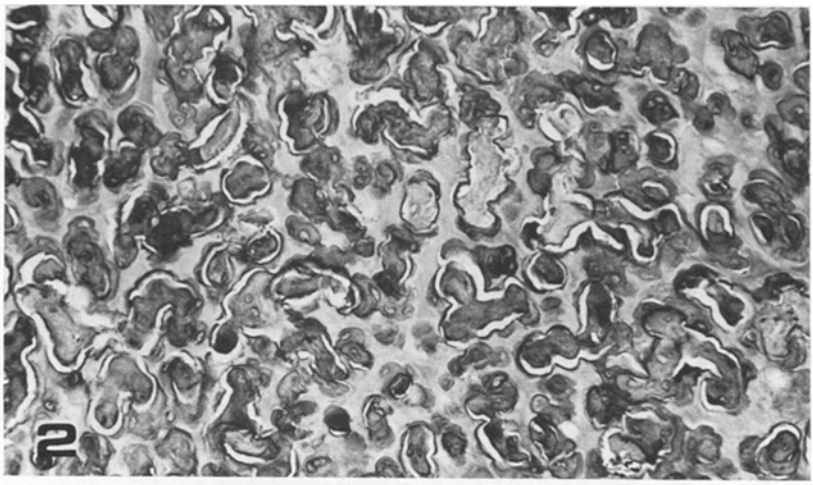


Fig. 1a—c. Roentgenograms of (a) skull, (b) upper and (c) lower limb

The costo-chondral synchondrosis of the rib appeared completely abnormal. It was essentially characterized by a large seriated zone in which clusters of hypertrophic chondrocytes predominated (Fig. 4). Large and irregular islands of calcified matrix were present and areas containing bone marrow cells, blood vessels and fibroblasts were recognizable in the not yet calcified cartilage (Fig. 5). However, the cartilage matrix was normal as regard to its staining properties with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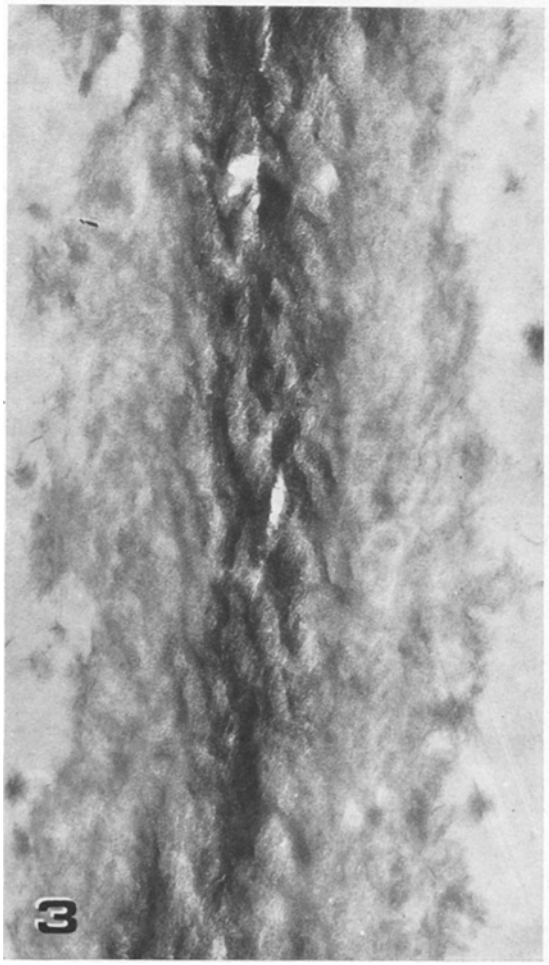
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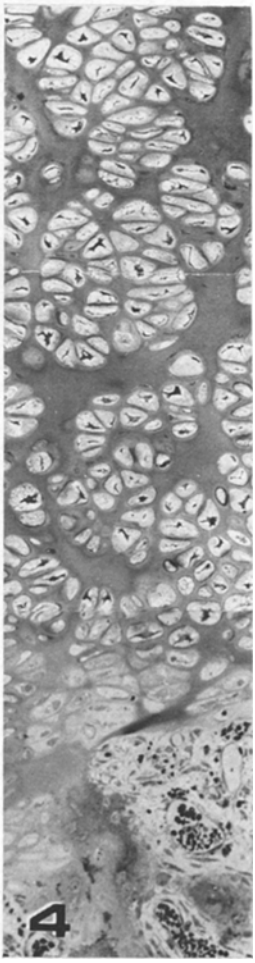
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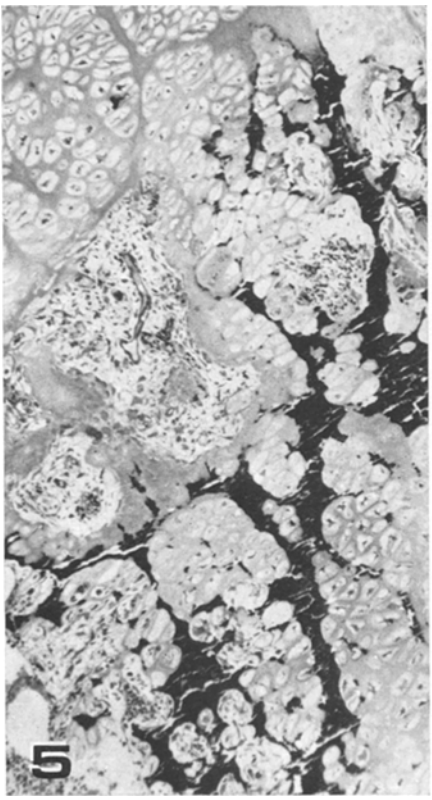

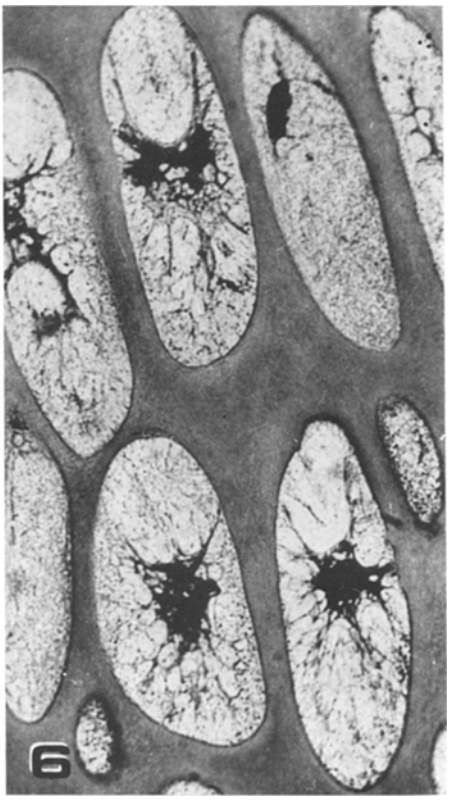

Fig. 2. Morphology of the osteo-cartilaginous trabeculae which completely obliterated the bone marrow cavity of the long bones. Hematoxylin-eosin, × 105

Fig. 3. Ultrastructure of an osteo-cartilaginous trabecula. The axial cartilaginous core appears more compact and electron-dense than the calcified bone matrix. Unstained, × 24000

Fig. 4. Costo-chondral junction: the increased number of hypertrophic and degenerate chondrocytes gives the cartilage a rickety appearance. Areas of primitive bone marrow are visible below. Azure II–Methylene blue, × 150

Fig. 5. Zone of calcification in the costo-chondral junction. Irregular calcified areas are present in wide zones of uncalcified cartilage. Islands of primitive bone marrow are also visible. Von Kossa, × 105

Fig. 6. Hypertrophic and degenerate chondrocytes of the costo-chondral junction. Azure II-Methylene blue, × 1050

PAS, alcian blue, colloidal iron and Mallory's method. The increased number of hypertrophic cells gave a rickety appearance to the epiphysis. The chondrocytes were not always placed in regular columns and in some cases their arrangement was distorted. However, they looked like normal hypertrophic and degenerating chondrocytes, although their lacunae sometimes contained an excess of fibrillar material (Fig. 6).

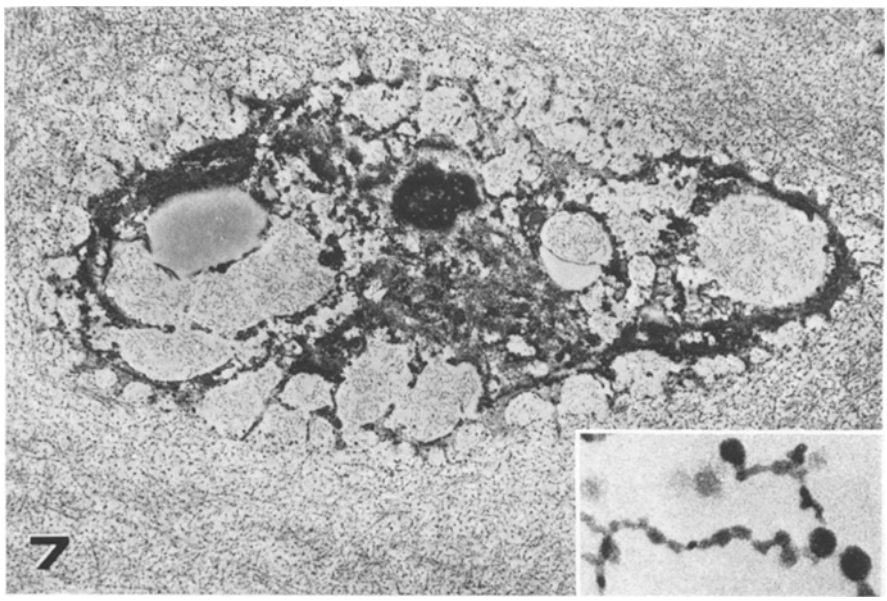

Fig. 7. Hypertrophic and degenerate chondrocyte of the costo-chondral junction. Many vacuoles are present in the cytoplasm. They contain a lot of beaded filaments, which are shown in detail in the inset. Uranyl acetate and lead citrate, × 7000 and, inset, × 110000

Under the *electron microscope,* these chondrocytes appeared highly vacuolated (Fig. 7) and the vacuoles contained very typical filaments having a thickness of about 65 Å and a characteristic beaded structure, with small and dense circular bodies, about 150 Å in diameter, present at their extremities (Fig. 7, inset). These filaments were sometimes found within vacuolated mitochondria and it was possible to observe that the vacuoles were often exocytosed, with consequent release of beaded filaments in the extracellular space (Fig. 7). Actually, beaded filaments were present in the territorial, but not in the interterritorial matrix.

The ultrastructure of the cartilage matrix did not seem substantially abnormal. It consisted of very thin, generally unbanded collagen fibrils, and small dense granules (Fig. 7). Electron-dense, roundish bodies often surrounded by a membrane were found in the matrix (Fig. 8). In some cases, these bodies were present within otherwise empty cellular lacunae. They were often placed near degenerating chondrocytes. Clusters of inorganic crystals were found within these bodies and in close relationship with them. The calcification process, where present, did not look different from that found in normal cartilage. However, it appeared to be greatly delayed because large areas of cartilage were left completely uncalcified.

Occasionally, abnormal collagen fibrils were present in proximity of the cellular lacunae. These fibrils were of irregular shape and thickness and showed a periodicity of about 2250 Å (Fig. 9). They were in some cases in close contact with bundles of irregularly arranged filaments which had the same morphology as that of

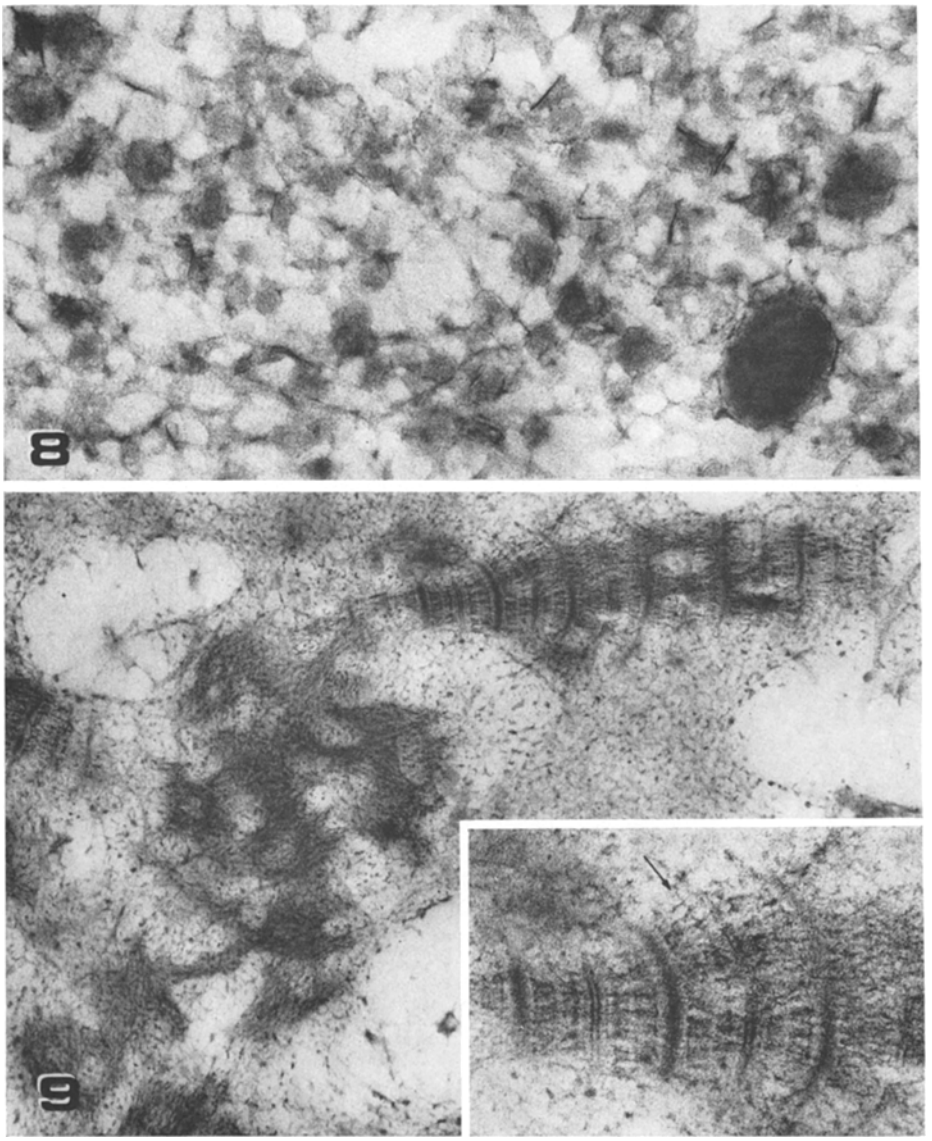

Fig. 8. Electron-dense bodies in an area of initial calcification of the cartilage. Uranyl acetate and lead citrate, × 75000

Fig. 9. Collagen fibrils of abnormal shape and thickness in the matrix of the cartilage. Note irregular bundles of electron-dense filaments similar to those which form the fibrils. Inset: detail of the collagen fibril shown on upper right: arrow points to a thin, branched filament which crosses the fibril. Uranyl acetate and lead citrate, × 46000 and, inset, × 96000

the filaments forming the fibrils (Fig. 9). Interestingly, some of these fibrils were crossed by branched filaments, whose branchings were oriented according to the axis of the fibrils (Fig. 9).

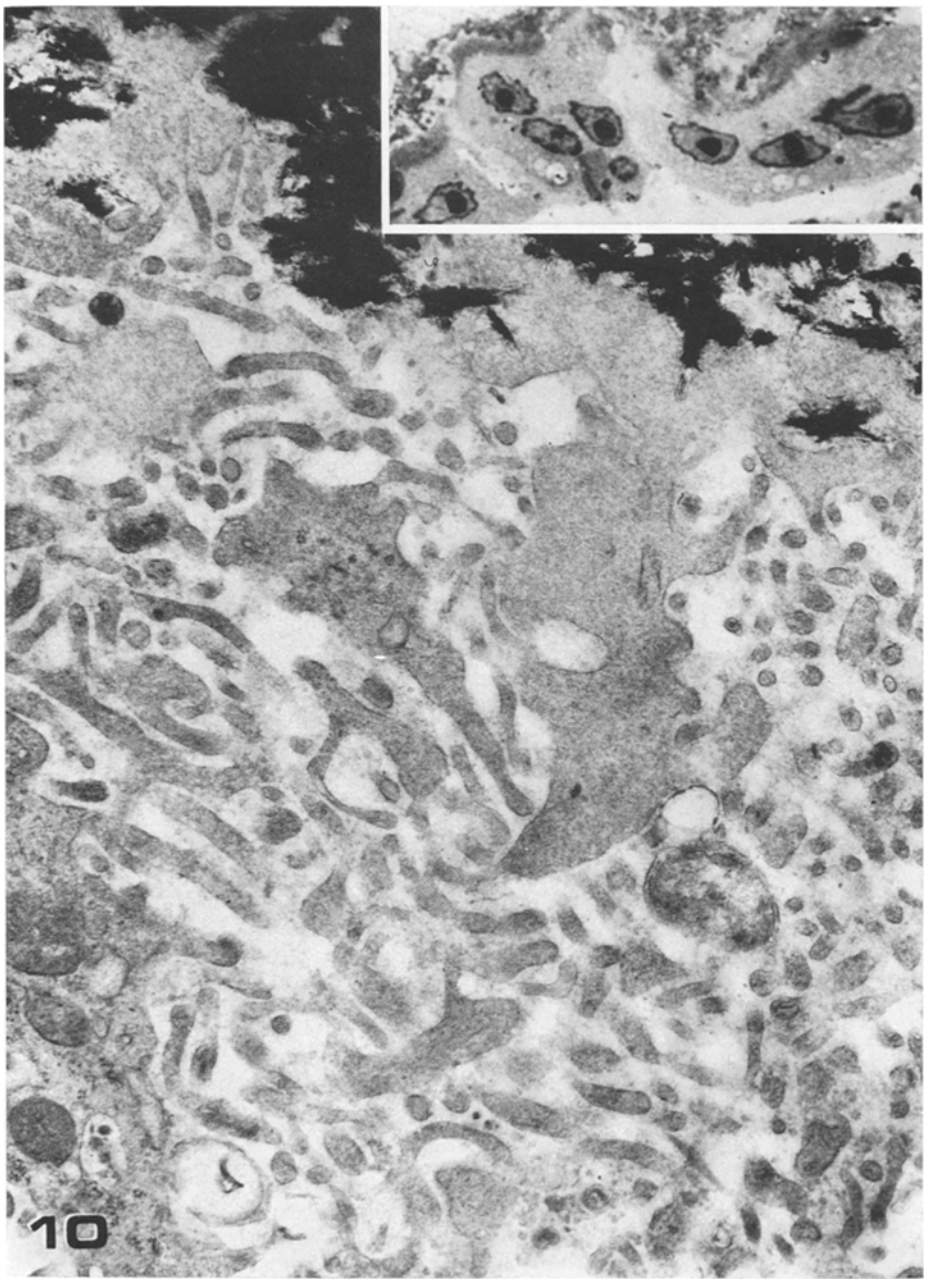

Fig. 10. Inset: group of osteoclasts along the border of an osteo-cartilaginous trabecula. Their morphology seems normal. Azure II-Methylene blue, × 1000. Detail of the brush border of an osteoclast as seen under the electron microscope: the brush is wider than in normal osteoclasts and no evidence of phagocytosis is visible. Uranyl acetate and lead citrate, × 27000

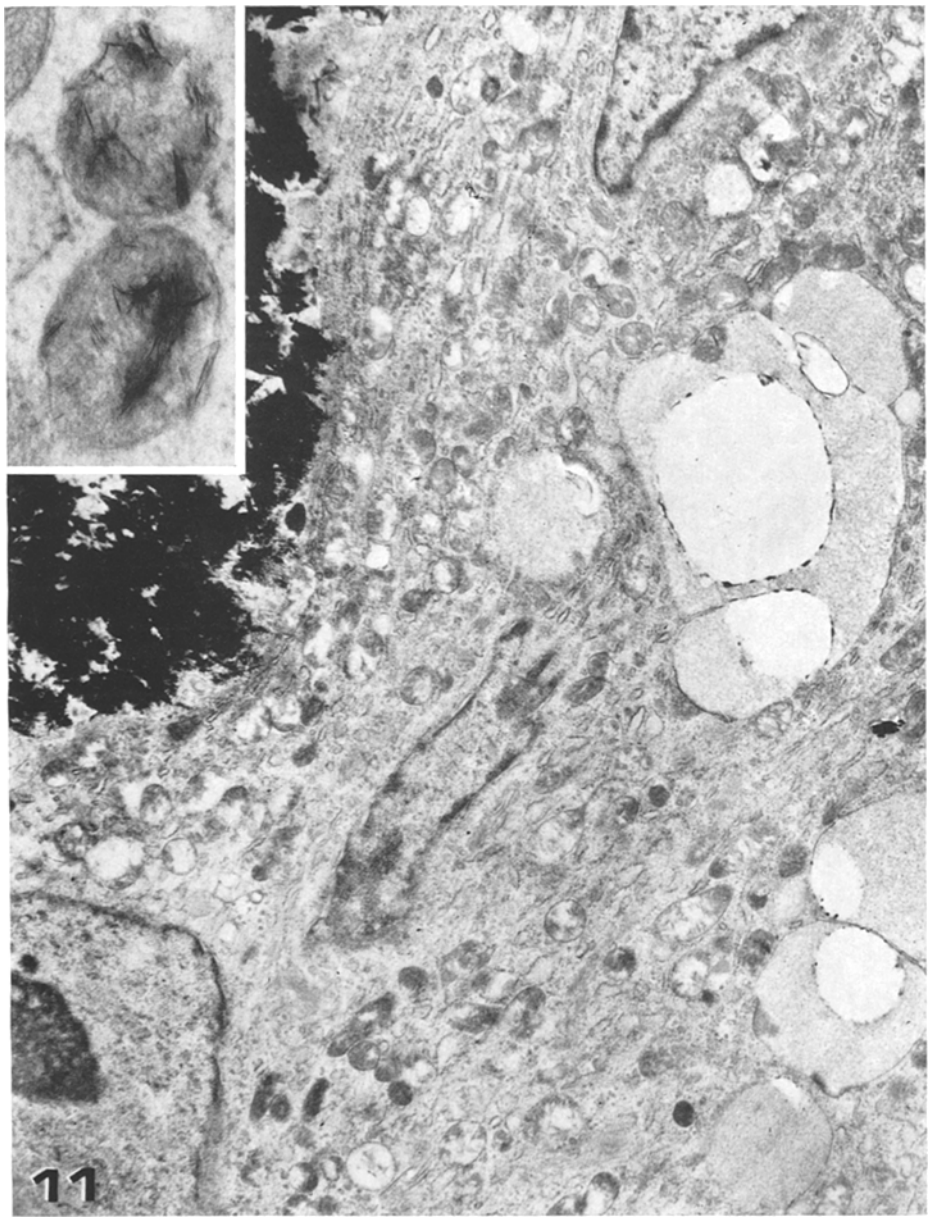

Fig. 11. Part of an osteoclast and of bone matrix adjacent to it. The brush border is lacking; the cytoplasm contains many mitochondria and large vacuoles. Three nuclei are visible. Inset: detail of osteoclastic mitochondria which contain inorganic crystals. Uranyl acetate and lead citrate, × 10000 and, inset, × 70000

Table 1. Water, Ca and P contents of organs from a case of osteopetrosis fetalis compared with those found in normal subjects. Values are reported as g/100 g of fresh tissue; bone values are reported as per cent of dry matter

Parts analyzed	Water		Calcium		Phosphorous	
	Normal[a] (%)	Sample (%)	Normal[b] (%)	Sample (%)	Normal[b] (%)	Sample (%)
Heart	73.7	85.9	0.0049–0.0150	0.0093	0.125–0.235	0.0285
Liver	71.5	80.6	0.0072–0.0094	0.0132	0.180–0.240	0.0771
Kidneys	79.5	86.6	0.0192	0.0046	0.170	0.0194
Bone	31.8	49.5	25.60	9.83	12.30	4.26

[a] Mitchell *et al.* (1945).
[b] Handbook of Biological Data, Ed. William Spector, W. B. Saunders Comp. Philadelphia and London, 1956.

No ultrastructural changes were found in the osteoblasts placed along the calcified osteo-cartilaginous trabeculae. Also the collagen fibrils of the osteoid borders appeared normal. However, they were loosely arranged and often formed large osteoid areas. The calcification process appeared delayed and often consisted of small clusters of loosely arranged crystals randomly scattered in the osteoid matrix. Moreover, wide zones of cartilage and bone matrix were left completely uncalcified.

Many osteoclasts were present along the calcified trabeculae. Under the light microscope, they showed a normal appearance (Fig. 10). On the contrary, under the electron microscope they appeared abnormal. In some cases, the brush border was excessively developed and very few inorganic crystals, if any, were present between its infoldings (Fig. 10). In other cases, the brush border was completely lacking (Fig. 11) and the osteoclasts were in direct contact with the calcified matrix without interposition of the ectoplasmic layer. In these cases, large vacuoles containing amorphous and filamentous material and electron-dense granules were present in the cytoplasm. Moreover, a lot of mitochondria were present. Interestingly, some of these mitochondria contained small clusters of inorganic crystals (Fig. 11, inset). No other evidence that this type of osteoclasts were engaged in reabsorption of calcified matrix was present.

The results concerning water, calcium and phosphorous content of the examined tissues are shown in Table 1. Water appears to be normally distributed in heart, liver and kidneys, while its content in bone is significantly higher than the normal value. In heart and liver calcium content seems to be normal, while in kidneys it is very low. However, phosphorous content of these three organs appears to be significantly lower than the reported normal values. Both calcium and phosphorous contents of bone are notably decreased, with a Ca/P molar ratio of 1.79.

Discussion

The clinical and pathological data found in the present case are all consistent with the diagnosis of osteopetrosis fetalis. One of the most severe change of the skeleton was the enlargement of the zone of balloned hypertrophic and degenerate

chondrocytes. This abnormality, which is typical of osteopetrosis fetalis (Herting and Liebegott, 1971; Jaffe, 1972; Schachenmann, 1948; Uehlinger, 1949), gave a rickety appearance to the sites of active endochondral ossification. The cartilage of this zone did not look abnormal with histochemical and histological techniques. On the contrary, electron microscopy showed that the chondrocytes and, at least to some extent, the matrix were atypical.

The chondrocytes appeared degenerate and their cytoplasm was almost completely occupied by large vacuoles. The nature and origin of these vacuoles have been recently established by electron microscope investigations of ageing rib and tracheal cartilage (Bonucci *et al.*, 1974; Dearden *et al.*, 1974; Dearden and Bonucci, in press). It has been reported that the vacuoles originate within degenerate mitochondria of degenerating chondrocytes. They contain beaded filaments completely similar to those found in the present case. The vacuoles fuse with the cell membrane, the filaments are exocytosed, and probably become components of the matrix.

Although the physiological role of these vacuoles and beaded filaments is not yet known, it seems obvious that their exceedingly high number in osteopetrosis fetalis is an index of cellular abnormality and, more specifically, of mitochondrial abnormality if it is accepted that vacuoles and beaded filaments firstly appear in degenerate mitochondria, as suggested by Dearden and Bonucci (in press).

The ultrastructure and histochemistry of the cartilage matrix did not seem to be abnormal. However, the occasional presence of anomalous collagen fibrils could be indicative of abnormal collagen secretion and/or fibril assembling, These anomalous fibrils were often in close connection with irregularly arranged filaments which could represent unaggregated tropocollagen molecules. The fibrils themselves appeared poorly aggregated. They were sometimes crossed by branched filaments. Although the nature of these filaments has not been established, it is attractive to speculate that they represent proteoglycan molecules engaged in fibril formation and assembling. This hypothesis is based on the fact that in normal cartilage proteoglycan molecules are bound to collagen fibrils and their proteic cores lie transverse to the long axis of the fibrils (Serafini-Fracassini and Smith, 1974). It has not been possible to establish if there was a direct relationship between the exceedingly high number of vacuoles and beaded filaments in the chondrocytes and the presence of these abnormal collagen fibrils in the matrix.

The process of calcification was greatly delayed in both cartilage and bone matrix. However, in both these tissues the calcified areas appeared qualitatively normal. The reduced Ca/P molar ratio and the high water content in bone could probably be explained by the presence of cartilaginous cores in many trabeculae and by a relative increase of the organic matter.

The earliest clusters of inorganic crystals were closely related to roundish, electron-dense bodies morphologically similar to the roundish matrix vesicles which have been described in normal cartilage (Anderson, 1968; 1969; Bonucci, 1967, 1970, 1971) and bone (Anderson, 1973; Bonucci, 1971), where they are considered to be the earliest locus of calcification (Bonucci, 1971). Matrix vesicles were not found in osteopetrosis so frequently as in normal cartilage and they were often contained within cellular lacunae, as reported in other pathological conditions of the cartilage (Bonucci *et al.*, 1974; Bonucci and de Matteis, 1968; Dearden *et al.*, 1974).

The structure of the osteoclasts was completely abnormal. They frequently showed complete or almost complete absence of brush border, a finding similar to that recently observed in *ia* rats (Marks, 1973; Schofield *et al.*, 1974). In other cases, the brush border was excessively developed. In both instances, the rate of bone resorption was strongly reduced or completely abolished. Large vacuoles were present in the cytoplasm of the osteoclasts. They could represent a further indication of lysosomal abnormality, clearly shown by the high intracellular acid phosphatase activity which is typical of the osteoclasts in osteopetrosis (Handelman *et al.*, 1964; Marks, 1973; Schofield *et al.*, 1974). Interestingly, clusters of inorganic crystals were sometimes present in osteoclastic mitochondria. Although it has been clearly established that mitochondria regulate the intracellular concentration of calcium ions (Bonucci *et al.*, 1973; Carafoli and Lehninger, 1971; Greenawalt *et al.*, 1964; Lehninger, 1970), the presence of intramitochondrial inorganic aggregates is not frequent in osteoclasts and when present they consist of roundish aggregates of amorphous and granular material (Gonzales and Karnovsky, 1961; Matthews, 1970). The presence of clusters of inorganic needle-shaped and filament-like crystals in mitochondria of osteopetrotic osteoclasts, while showing that these cells have to some extent resorption activity, could be a further demonstration of the abnormality of the osteoclasts in osteopetrosis.

All these findings show that the principal abnormalities which occur in osteopetrosis fetalis concern both the chondrocytes, the cartilage matrix, and the osteoclasts. In areas of endochondral ossification, there is excessive formation of hypertrophic and degenerate chondrocytes which appear vacuolated and contain an exceedingly high amount of beaded filaments. Moreover, abnormal collagen fibrils are sometimes present. The calcification process is greatly delayed, probably in connection with a reduced rate of matrix vesicle formation, but it appears qualitatively normal. Also the osteoclastic resorption activity is greatly delayed and reduced, or completely lacking.

The accumulation of osteo-cartilaginous trabeculae in marrow spaces seems to be dependent on the combined effect of the altered structure of the chondrocytes, the delayed calcification of the matrix, and the reduced rate of osteoclastic resorption.

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