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The Cloverleaf Skull Syndrome

Histological, Histochemical and Ultrastructural Findings*

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Summary. A histological, histochemical and ultrastructural study of a case of the cloverleaf skull syndrome is presented. It involved pronounced trilobed deformation of the skull associated with synostosis of the lambdoidal and sagittal sutures, hydrocephalus, downward displacement of the ears, hypertelorism, exophthalmus, a sunken nasal root and antimongoloid slant of the eyes. Shortening and bending of the limbs, narrowing of the chest, and deformation of the feet completed the picture.

Histologically, evident abnormality of the cartilage was found both in the sphenoidal and vertebral bodies and in the chondro-osseous junctions of the ribs. These zones were not seriated and contained only rounded, seemingly degenerating, disarrayed chondrocytes. The intercellular matrix was strongly PAS-positive. It stained irregularly and in general less deeply than in normal subjects after treatment with Toluidine blue, Alcian blue and colloidal iron. Moreover, both the cartilage and bone matrix contained collagen fibrils of highly variable thickness. In both these matrices the process of calcification was irregular and slightly retarded.

It is suggested that the cloverleaf skull syndrome essentially depends upon a chondrodystrophic condition which involves the base of the skull and may be generalized, as in the present case, or limited to one skeletal area, as sometimes reported in the literature.

The so-called "Kleeblattschädel" or "cloverleaf skull" syndrome is a remarkable congenital skeletal dysplasia. It was first recognized by Holtermüller and Wiedemann in 1960 in a four-and-a-half month old child. Its chief characteristic is the grotesque, trilobed shape of the skull associated with severe internal hydrocephalus. By examining the German literature, Holtermüller and Wiedemann were able to identify twelve additional cases, previously classified as chondrodystrophic hydrocephalus. These German Authors grouped all 13 cases within a clearly defined syndrome, whose main features they listed as: 1. trilobed skull with downward displacement of ears; 2. facial deformities, chiefly involving the orbits, nasal root and jaw; 3. micromelia and abnormal vertebral column; 4. x-ray evidence of cloverleaf shape of the skull and of hydrocephalus; 5. poor prognosis.

Fifteen more cases have been reported since 1960 (Liebaldt, 1964; Comings, 1965; Angle *et al.*, 1967; Moscatelli *et al.*, 1968; Wollin *et al.*, 1968; Feingold *et al.*, 1969; Nawalkha and Mangal, 1970; Partington *et al.*, 1971; Schuch and Pesch, 1971). As in the 13 preceding cases, however, attention has chiefly been given to clinical and radiological data. Anatomo-pathological and histological analysis has been extremely scanty. Liebaldt (1964) carried out histological studies on various skeletal segments from the case described in 1960 by Holtermüller and

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Wiedemann and from one additional case, though here the material had been badly damaged by prolonged soaking in formalin. He reported the abnormal presence of abundant fibrous connective tissue and persistence of blood vessels with embryonal characteristics within enlarged bone-marrow spaces. These lesions were attributed to fibrous bone dysplasia with angiomatosis. On the other hand, Case 3 of Partington *et al.* (1971) showed abnormal endochondral ossification, particularly in the long bones of the extremities, seen chiefly in the disarrangement and distortion of chondrocyte columns. As far as we know, there have been no other histological investigations on cartilage and bone of subjects with cloverleaf skull syndrome .

We have recently had the opportunity of examining a female newborn with this syndrome. We feel it useful to report this additional case not only because such a rare disease arouses spontaneous interest, but also because we have been able to compare the clinical and radiological features of many skeletal segments with their histology, and because, for the first time in the literature, this syndrome has now been investigated histochemically and under the electron microscope.

Case Report

M. D., female newborn, 5th child of a healthy woman of 34. Weight at birth 2100 g. Normal delivery after a 40-week pregnancy complicated by hydramnios. The father, mother and 4 brothers are all healthy. Knowledge of their previous history is limited, but it is known that the parents are not related and that there is no record of skull deformity, short stature or other gross skeletal abnormalities in the family.

M. D. died 5 hours after birth; respiration was very irregular and superficial, and was associated with rales in the chest. She had a markedly deformed skull, which was enlarged and trilobed in shape. Her chest was narrow and her limbs obviously abnormal. These lesions were provisionally classified as achondroplasia with hydrocephalus.

Post-mortem *x-ray examination* showed a typical "cloverleaf" skull (Figs. 1, 2) with pronounced acrocephaly (Fig. 2) and bulging temporal regions (Fig. 1). The bulging temporal regions were incompletely ossified, with a large fault in their most prominent part (Fig. 1). In lateral projection, the roentgenogram showed that the antero-posterior diameter of the skull was unduly small, while its height was disproportionately great (Fig. 2). The cranial base was clearly deformed: the anterior and middle fossae were too small, while the posterior fossa was, by comparison, too large. Ossification of the vault was rudimentary. Only the back of the skull was normally ossified. It was oriented vertically and had an exostotic-like thickening above the posterior fontanelle.

The height of the vertebrae was abnormally small, while the intervertebral spaces were unduly large. The ribs were short and thick. The pelvis was achondroplastic in shape, with broad iliac wings and pelvic width exceeding depth. The limbs (Fig. 1) were short, curved and slightly thicker than normal. Their metaphyses were rather irregular.

Post-Mortem Examination (autopsy n^0 42244 of the Institute of Morbid Anatomy, University of Rome). Only major necroscopic features are reported.

The skull was severely deformed. Its vertical and transverse diameter were large, while the antero-posterior diameter was small. The skull had a trilobed shape due to swelling in both temporal areas and in the region of the vertex. The ears were abnormally low. There was hypertelorism, exophthalmos and antimongoloid slant of the eyes. The nasal root was sunken. The vertex of the skull appeared completely membranaceous. The parietal bones were displaced backwards and were vertically oriented. They formed the back of the skull and looked like a large occipital squama. Both the sagittal and lambdoidal sutures were synostosed. The posterior fontanelle was ossified. The wall of the skull bulged outwards on this side, forming an exostotic-like bulge. The anterior fontanelle was wide open. It extended forward in a widely diastased metopic suture, and sideways into the coronal suture, which, as a result,

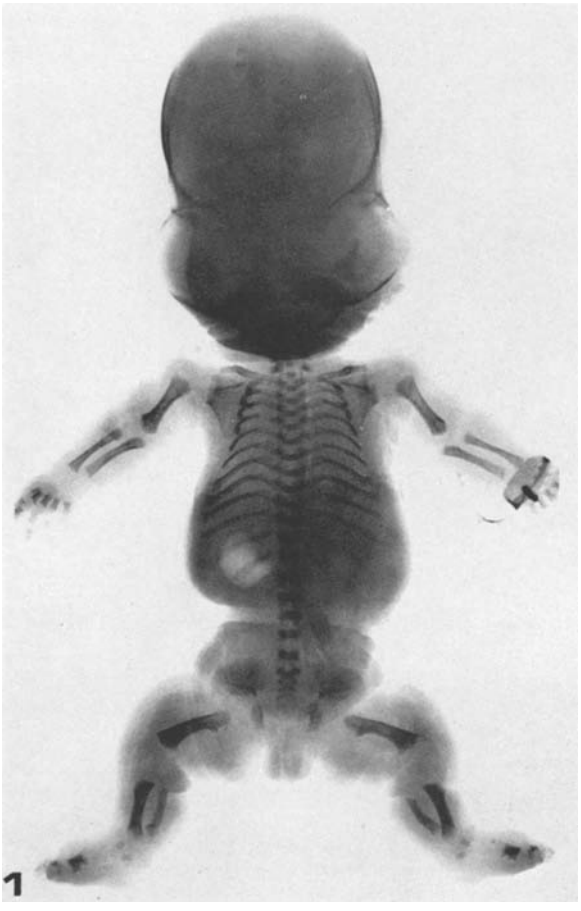


Fig. 1. Roentgenogram showing general body configuration. Note the typical trilobed shape of the skull

was almost completely diastased. The temporal squamae bulged outwards and were displaced downwards. The vertex of the bulges was not ossified and seemed to be continuous with a wide-open sphenoidal fontanelle.

The base of the skull had a small antero-posterior diameter, chiefly because of the small size of the anterior and middle fossae.

The limbs were short and the legs extrarotated. There was mild talipes equinovarus.

Microscopic Examination. Osseous and cartilagineous specimens were taken from various parts of the skeleton, including the occipital zone of the skull, the sphenoidal body, the vertebral column and the condro-osseous junctions of the ribs. Relatives refused to allow the long bones of the limbs to be withdrawn. All the specimens were fixed in 4% formalin buffered to pH 7.2 according to Millonig (1962). Small fragments including both cartilage and bone were taken from the chondro-osseous junctions of the ribs and postfixed in 1% OsO_4 buffered to pH 7.2 as above. These small fragments were used for electron microscopy.

Specimens for optical microscopy were embedded in paraffin. Those including large calcified zones were previously decalcified with 2% formic acid or with EDTA. Sections were stained by one of the following methods: Hematoxylin-eosin for routine examination; Mallory method for collagen; periodic acid-Schiff (PAS) method for glycoproteins; Toluidine blue, Alcian blue and colloidal iron (all at pH 2.5) for acid polysaccharides.

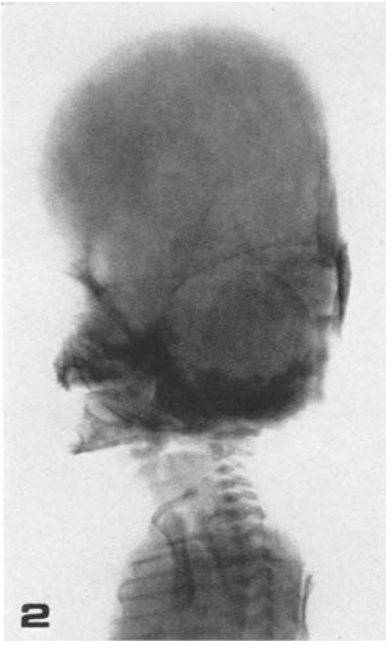


Fig. 2. Roentgenogram of the skull, lateral view

Specimens for electron microscopy were embedded in Araldite after Acetone dehydration. Some sections were examined unstained, others after treatment with uranyl acetate and lead citrate.

Results of Optical Microscopy

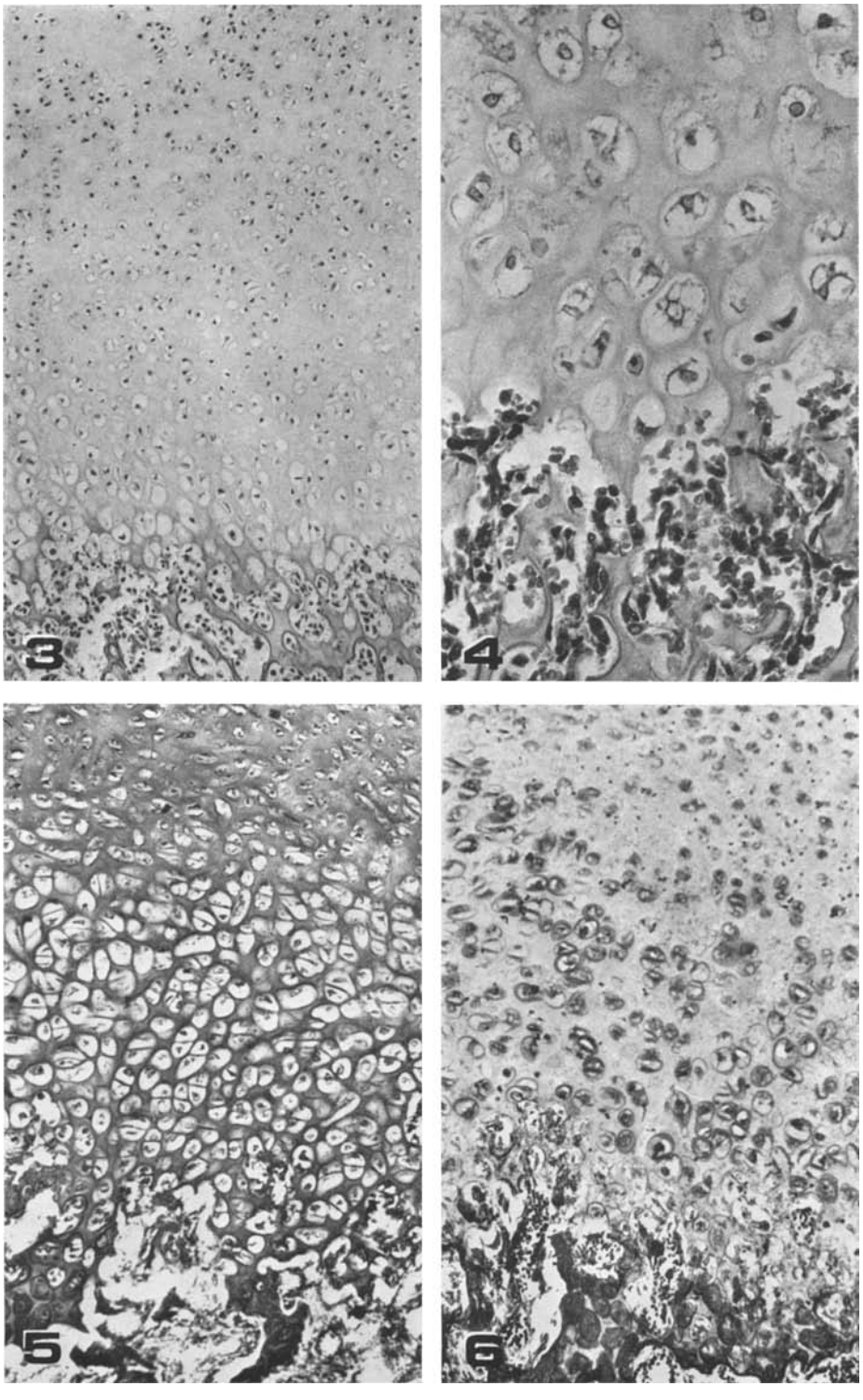
Fig. 3 shows the histologic picture typical of the *endochondral ossification* found in all the bones examined. Both in the sphenoidal and vertebral bodies and in the chondro-osseous junctions of the ribs, the clearest abnormality is the absence of seriated cartilage in the zones of ossification (Figs. 3—6). The cartilage area between the so-called resting cartilage and the zone of provisional calcification does not contain columns of seriated chondrocytes as in normal subjects. Only roundish, slightly hypertrophic, seemingly degenerating chondrocytes are present (Figs. 3, 4). They are irregularly distributed through the cartilage matrix. However,

Fig. 3. Front of calcification in chondro-osseous junction of the ribs. Note absence of seriated cartilage and presence of poorly formed, irregularly oriented calcified trabeculae. Hematoxylin-eosin, × 100

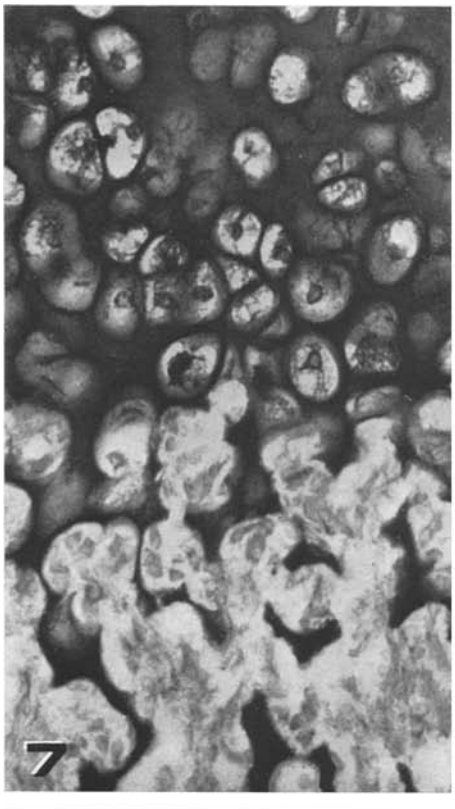
Fig. 4. Front of calcification in chondro-osseous junction of the ribs. Seriated cartilage is replaced by roundish, slightly hypertrophic, seemingly degenerating chondrocytes. Hematoxylin-eosin, × 260

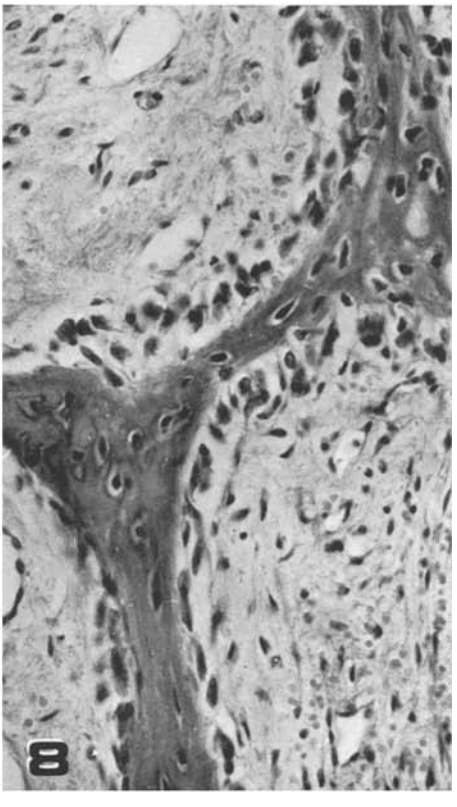
Fig. 5. Front of calcification in the sphenoidal body. Chondrocytes are like those in costal cartilage. The intercellular matrix is homogeneously PAS-positive. PAS, × 100

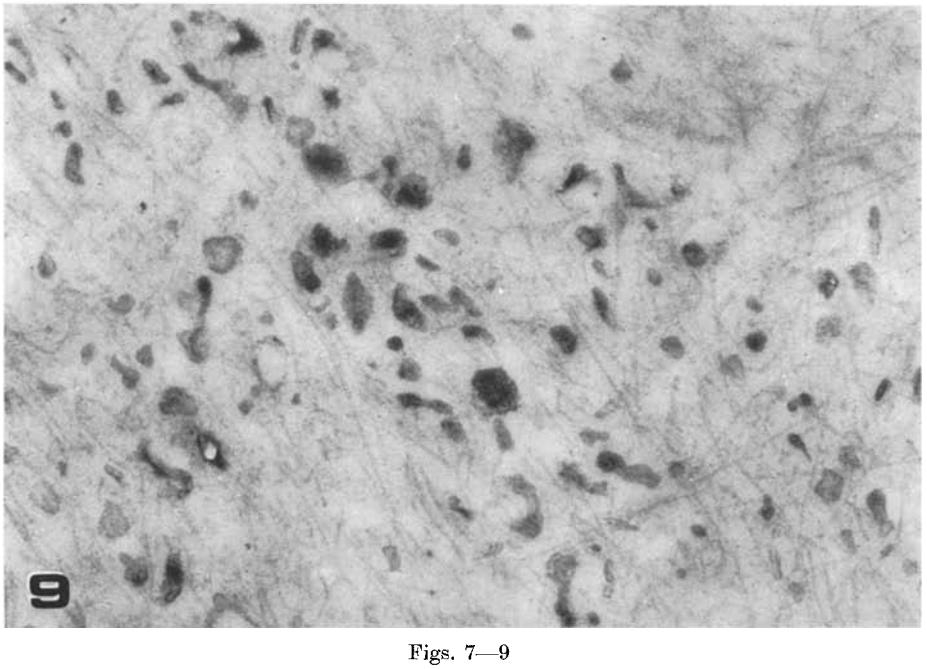
Fig. 6. Front of calcification in a vertebral body. Only the border of the chondrocyte lacunae is stained. Alcian blue, × 100



Figs. 3—6







Figs. 7—9

they sometimes form a layer easily distinguishable from the adjacent zones occupied by resting cartilage on one side and calcifying cartilage on the other (Fig. 5). In this last zone, the calcified cartilaginous trabeculae are poorly formed, highly distorted and very irregularly oriented and arranged (Figs. 5, 6). Moreover uncalcified cartilage is sometimes in direct contact with bone-marrow cells and blood vessels (Fig. 4). The primary osseous spongiosa is also very irregular. The ossification process does not, however, seem abnormal, even if osteoid borders are often wider than normal.

Both cartilage matrix and osseous trabeculae were homogeneously PAS-positive (Fig. 5). No glycogen was detectable in hypertrophic, degenerating chondrocytes. Less homogeneous results were obtained by staining with Toluidine blue, Alcian blue and colloidal iron. In some cases the border of the cellular lacunae was deeply stained and metachromatic while the intercellular matrix was almost wholly unstained and very faintly metachromatic (Fig. 6). In others, the cartilage matrix was evenly stained and metachromatic and there was no apparent difference between territorial and interterritorial matrix (Fig. 7).

The process of *membranous ossification* studied in the occipital squama did not seem abnormal, although the ossified layer was unusually thin. The osseous trabeculae were normally structured and their surface often lined by large osteoblasts (Fig. 8). The synostosed sutures consisted of numerous and rather large bone trabeculae, but no abnormal characteristics were identifiable in them.

The osseous trabeculae of the occipital squama were PAS-positive, slightly metachromatic and alcianophilic and had low affinity for colloidal iron.

The *periosteal ossification* studied in the osseous ribs revealed the formation of rather thin and irregular trabeculae. The outer compacta was thin and occasionally discontinuous, but the ossification process as a whole did not seem to differ appreciably from normal, except that the trabeculae sometimes had unusually thick osteoid borders.

Staining for glycoproteins and acid polysaccharides gave no abnormal results.

Electron Microscopy

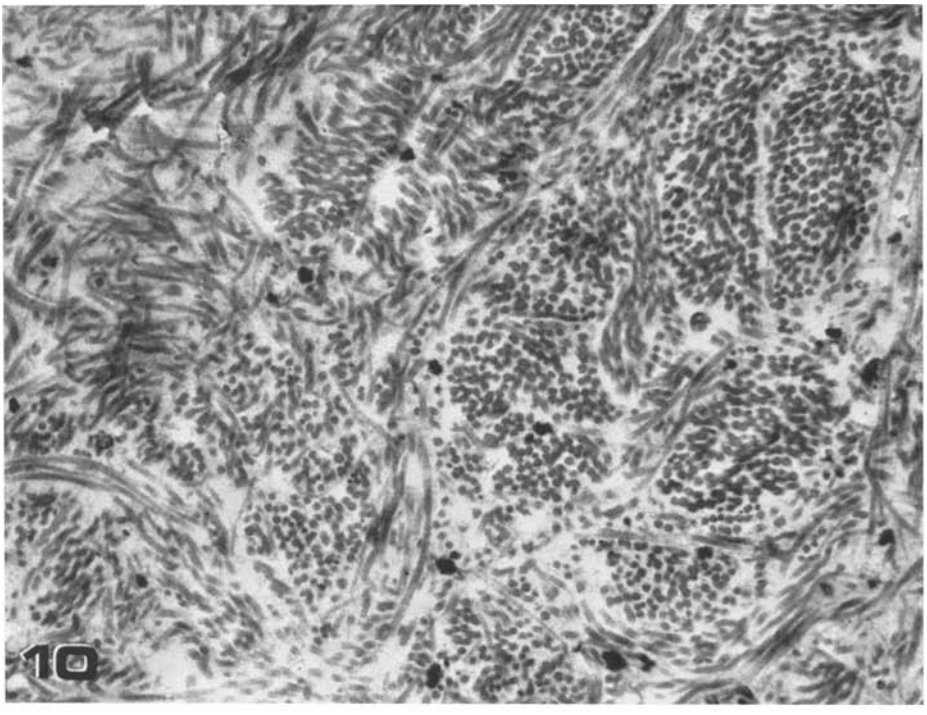
Electron microscopy had to be restricted to the cartilaginous and osseous organic matrices of the chondro-osseous junctions of the ribs, and to the calcification process in them, because the cells showed initial changes due to post-mortem autolytic processes and were therefore judged to be unreliable for ultrastructural investigation.

The uncalcified *organic matrix in the cartilage* adjoining the zone of provisional calcification consisted of an irregular network of thin collagen fibrils separated by large interfibrillary spaces. This matrix did not look very unlike that generally

Fig. 7. Front of calcification in a vertebral body. The intercellular matrix is homogeneously stained. Alcian blue, × 260

Fig. 8. Membranous ossification of the skull. A bone trabecula lined by large osteoblasts. Hematoxylin-eosin, × 260

Fig. 9. Cluster of osmiophilic, membrane-bounded bodies in the cartilage matrix near the calcification front. Uranyl acetate and lead citrate, × 60000



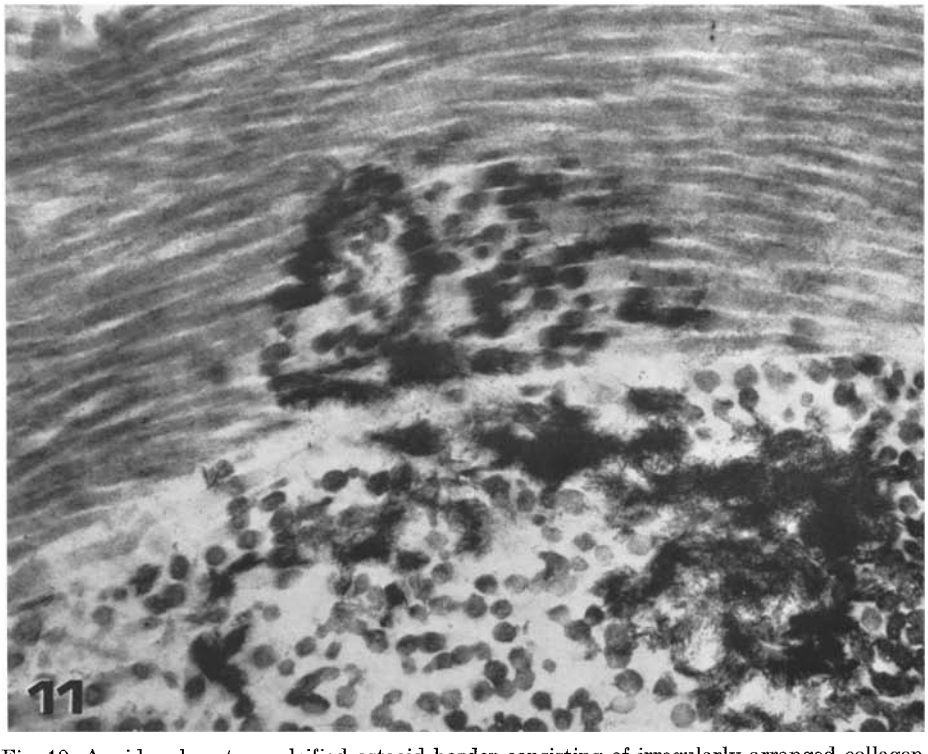
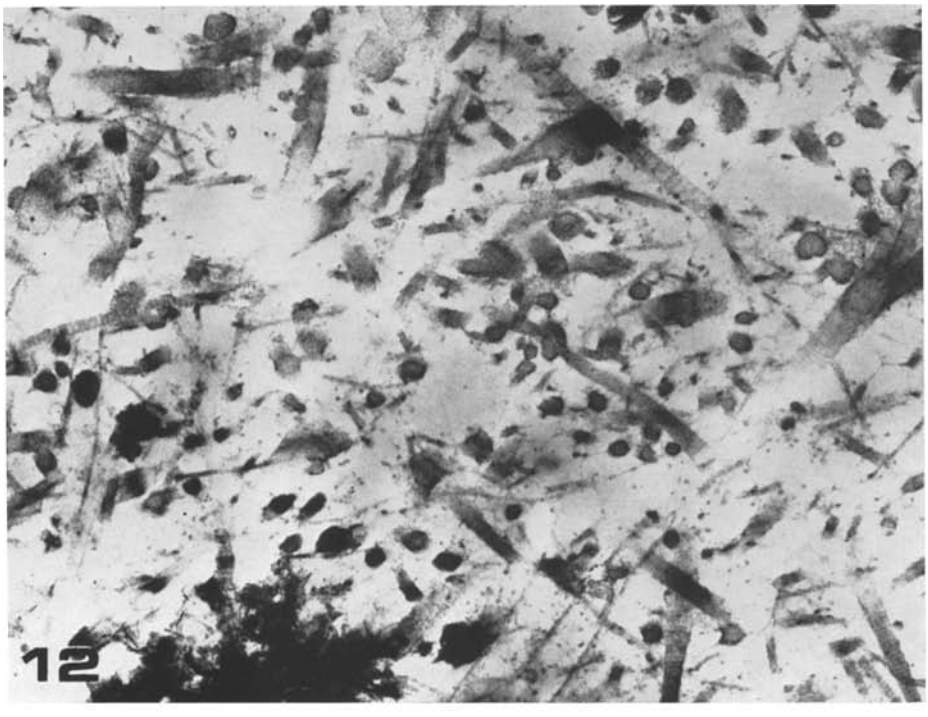


Fig. 10. A wide, almost uncalcified osteoid border consisting of irregularly arranged collagen fibrils. The black spots are areas of early calcification. Uranyl acetate and lead citrate, × 18000

Fig. 11. Osteoid border with collagen fibrils arranged in lamella-like structures. The collagen period is not clearly visible. Areas of initial calcification at the center and lower right. Uranyl acetate and lead citrate, × 54000



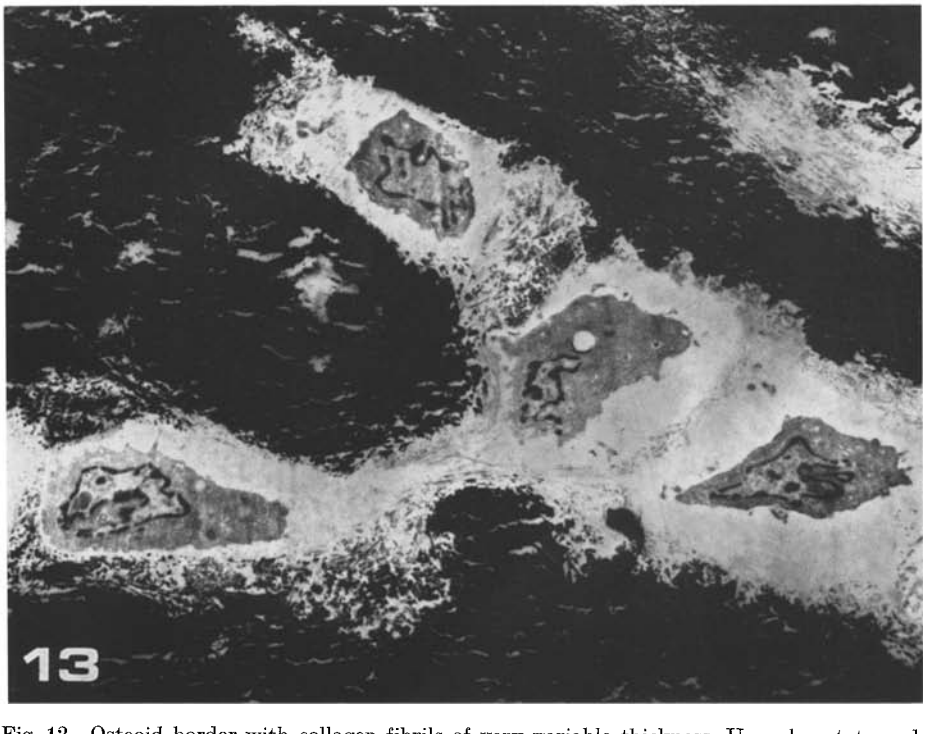


Fig. 12. Osteoid border with collagen fibrils of very variable thickness. Uranyl acetate and lead citrate, $\times 45000$

Fig. 13. Periosteal bone. Large, intercommunicating osteocytes are shown. Uranyl acetate and lead citrate, $\times 4000$

found in normal cartilage near the zone of provisional calcification. The only differences seemed to be the unusually wide range of thickness of collagen fibrils. There were also unusually large numbers of interfibrillary, osmiophilic, roundish, membrane-bound bodies (Fig. 9), some containing small clusters of apatite crystals.

The uncalcified *organic matrix of bone* was rather irregularly distributed. The osteoid borders were sometimes of normal thickness (i.e., no thicker than 2 μ) and sometimes far above it.

The collagen fibrils in the osteoid matrix were often irregularly oriented and loosely arranged (Fig. 10). At other times, closely adjacent collagen fibrils ran roughly parallel and formed lamella-like structures (Fig. 11). In most cases, the periodic banding of these fibrils was clearly visible, but in others it was not. In this case the fibril surface appeared finely granular, as if coated by an amorphous substance masking the period (Fig. 11). In other areas, usually those near the zone of provisional cartilage calcification, the collagen fibrils were of very variable thickness (Fig. 12). Fibrils about 100 Å thick interwove irregularly with fibrils about 800 Å thick and with others whose thickness varied along the whole range between 100 and 800 Å.

The *calcification process* did not differ substantially from that found in normal cartilage and bone. One characteristic of the early stage of the process was the formation of roundish clusters of needle-shaped crystals irregularly scattered through the organic matrix. These clusters later increased in size by addition of other crystals and gradually became more elongated. Adjacent crystal clusters then coalesced and the organic matrix calcified completely. However, areas of uncalcified matrix of varying size were occasionally left isolated within calcified matrix. In bone, these uncalcified areas frequently linked adjacent osteocytes with the formation of a labyrinth-like pattern within calcified matrix (Fig. 13).

Discussion

The definitive diagnosis of "cloverleaf skull" (Kleeblattschädel) syndrome was based on clinical, radiological and anatomo-pathological findings, especially the typical trilobed shape of the skull with downward displacement of the ears, hydrocephalus and the achondroplastic-like changes in the long bones.

The etiology of the syndrome is still unclear. The striking skull deformation has directed attention chiefly to the premature closure of some cranial sutures, particularly the coronal and lambdoidal ones. The trilobed shape of the head, the hydrocephalus, and the deformation of the face have been attributed to intrauterine synostosis of cranial sutures, by analogy with other cranial dysplasiae, such as Crouzon's disease and acrocephalosyndactily (Feingold *et al.*, 1969). The disease has therefore been thought to be mainly a result of damage to the mechanism of membranous ossification (Angle *et al.*, 1967), which may lead to: a) premature closure of sutures; b) secondary morphological adaptations of the skull chiefly due to expansion of the encephalus; c) abnormal development of the encephalus, which is forced to develop according to the direction of less intense bone constriction (Ascenzi, 1957). As regards the cloverleaf skull syndrome, it has been suggested that this anomalous membranous ossification may derive from the persistence of embryonic vascular tissue (primitive osteohemangioma) in the cranial vault (Liebaldt, 1964).

Recently, Burkardt (1970) has suggested that the clover-leaf-like deformation of the skull is primarily due to improper ossification of the cranial base consequent on an abnormal process of endochondral ossification.

This is in agreement with an investigation by Moss (1959) which seems to show that the premature closure of the cranial sutures is symptomatic of an abnormal growth of the base of the skull. On this view, the synostosis of the coronal suture is due to a spatial abnormality of the small wings of the sphenoid, and the synostosis of the sagittal suture to abnormality of the cribriform plate in the ethmoid and of the crista galli. Moss (1959) considers that the abnormal development of these parts of the base of the skull causes abnormal tension on the dura mater —chiefly on the falx cerebri and on the tentorium cerebelli—so that the neurocranium cannot develop normally according to the requirements of the growing brain. In this connection it is interesting that our investigation showed that the membranous ossification of the skull was histologically normal, while the endochondral ossification of the sphenoidal body was clearly abnormal.

It must not be forgotten that, as stressed by Partington *et al.* (1971), trilobed deformation of the skull is sometimes an isolated skeletal abnormality. This does not seem compatible with the hypothesis that the cloverleaf skull syndrome is to be considered a generalised chondrodysplastic disease. However, the cranial base has never been examined histologically in these cases. It is therefore uncertain whether the reported cases of isolated skull deformation are the result of an abnormal process of membranous ossification leading to premature closure of the sutures, or to localised chondrodysplastic lesion of the cranial base, as suggested by Moss (1959).

In the present case, a clear indication of a generalised chondrodysplastic process is provided by the complete lack of organization in the seriated zone of calcifying cartilage both in sphenoidal and vertebral bodies and in the chondro-osseous junctions of the ribs. The roundish, vacuolized, disarranged chondrocytes found in these skeletal segments near the zone of calcification and the low amount of ground substance interposed between cells are clear evidence of abnormal cartilage proliferation and calcification.

Chondrocyte morphology seems to be the same as that usually described in the epiphyseal cartilage of achondroplastic subjects (see Potter, 1953; Weinmann and Sicher, 1955; Rubin, 1964).

The histochemical properties of the cartilage matrix too seem to be analogous to those found in achondroplasia. In fact, both in this disease (Stanesco *et al.*, 1966) and in the present case, the cartilage matrix of the zone approaching the front of calcification is strongly PAS-positive, whereas the stain reactions for acid mucopolysaccharides are variable, but in most cases less pronounced than in normal subjects.

It does, however, seem that the cloverleaf skull syndrome is distinct from achondroplasia. Thus, synostosis of cranial vault sutures are not reported in achondroplastic subjects. Moreover, according to Rimoin *et al.* (1970), the literature concerning endochondral histopathology in achondroplasia is misleading. These authors state that a heterogeneous group of chondrodystrophies has wrongly been grouped under the heading achondroplasia and that, if only true achondroplastic subjects are considered, they have regular, well organized endo-

chondral ossification. Ponseti (1970), too, reports that the histological picture of the iliac crest is normal in achondroplasia, although he finds abnormal clustering of chondrocytes and derangement of the hypertrophic zone in the upper fibular head.

The need for further investigation is readily apparent. In the meantime Partington *et al.* (1971), who accept these recent views on achondroplasia, have included the cases of cloverleaf skull syndrome which involve generalised chondrodysplastic skeletal lesions within so-called thanatophoric dwarfism, recently described by Maroteaux *et al.* (1967). This expression refers to a form of dwarfism producing early death and characterized by severe micromelia, narrowing of the thorax, flattening of the vertebrae and relative enlargement of the head.

There is no doubt that, from a clinical and histological point of view, the present case could be considered a case of thanatophoric dwarfism, in line with the classification of Partington *et al.* (1971). But it is clear that thanatophoric dwarfism cannot be held to include all cases of cloverleaf skull syndrome, those without generalized skeletal lesions falling far outside the accepted definition of thanatophoric dwarfism. Besides, this label contributes little towards an explanation of the etiologic and pathogenetic factors active in cloverleaf skull syndrome.

Our results offer no radical solution to this problem but they do seem to provide strong evidence of the presence of a chondrodysplastic process in subjects with cloverleaf skull syndrome. The abnormal appearance of chondrocytes, the strong PAS-reactivity of cartilage matrix and the variation in results obtained after staining with Toluidine blue, Alcian blue and colloidal iron suggest an abnormal structure of the organic matrix and, in particular, an abnormal glycoproteic and mucopolysaccharidic composition. The abnormal thickness of the collagen fibrils, sometimes found in both cartilage and bone, could have a similar meaning. It is in fact well known that the nucleation and assembly of collagen fibrils is strictly dependent on the concentration and type of mucopolysaccharidic substances present at sites of collagen secretion (Lowther, 1963; Wood, 1964; Lowther *et al.*, 1970). An abnormal composition of the organic matrix could also explain the slightly delayed calcification of the bone matrix and the persistence of large, intercommunicating osteocytes.

In the present case, the abundant clusters of membrane-bound, osmiophilic, roundish bodies found in the cartilage matrix deserves special mention. These bodies have recently been described as normal constituents of epiphyseal cartilage (Bonucci, 1967, 1970, 1971; Matukas and Krikos, 1968; Anderson, 1969; Kimura, 1969; Anderson *et al.*, 1970; Smith, 1970; Schenk *et al.*, 1970; Thyberg and Friberg, 1970; Riede *et al.*, 1971). They contain a rich enzymatic endowment (Ali *et al.*, 1970, 1971; Matsuzawa and Anderson, 1970; Göthlin and Ericsson, 1971) and might have lysosome-like properties (Thyberg and Friberg, 1970; Thyberg, 1972). They are engaged in the early stage of the calcification process and the first inorganic crystals are found within them (Bonucci, 1967, 1970, 1971).

Many more of these structures have been found in the cartilage in the present case then in normal cartilage. This seems to stand as further evidence of the abnormal structure of epiphyseal cartilage in cloverleaf skull syndrome.

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