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The influence of stress and strain in the early development of shaft bones

An experimental study on the chick embryo tibia

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Summary. In chick embryos from stage 23 to stage 27 the whole presumptive zeugopod – or its pre-axial (tibial) portion only – was proximodistally and dorsoventrally inverted by turning it 180° round the anteroposterior axis of the limb bud.

Development of the reoriented blastema of the tibia was consistently retarded and variously reduced: this skeletal piece appeared shorter and relatively thicker than the controlateral normal tibia. Chondrification, progress of differentiation of the cartilaginous model, onset and gradual spreading of the ossification processes were considerably delayed.

Often the diminutive tibia underwent a degree of bending or angulation of up to 90°–100° in the sagittal plane. In these bent tibiae – obviously developing under abnormal conditions of intrinsic and extrinsic mechanical stresses – cell hypertrophy appeared greatly retarded or hindered in sites of the diaphysial cartilaginous core which were presumably subjected to strong longitudinal compression. No rigorous temporal and topographical relationships were observed between chondrocyte hypertrophy and onset of perichondral osteogenesis. Apparently, a direct contact between hypertrophic cartilage and perichondrial cells was not strictly required to prompt osteogenesis; this process, in fact, often involved areas of the perichondrium enveloping parvicellular cartilage. Radial pressures exerted by the overstretched outer layer of the perichondrium, or periosteum, on the subjacent prospective osteogenous layer reduced or prevented the deposition of bone. Conversely, radial stretching of the inner layer of the perichondrium, or periosteum, considerably enhanced cell proliferation, blood vessel formation, differentiation of osteoblasts and formation of bone matrix.

Key words: Chick embryo – Cartilage hypertrophy – Diminutive long bone – Mechanical strain and bone formation – Perichondral ossification

Introduction

In the course of an experimental investigation on determination of the site of development of joints in the hind limb of chicken embryos (Amprino 1981, 1983), dorso-ventral and proximo-distal inversion of the entire zeugopod, or of its pre-axial (tibial) portion only, was carried out.

The tibiae of the operated right limbs consistently appeared shorter, relatively thicker than the control bones and often bent or angulated in the sagittal plane. Histological study of the diminutive tibiae showed that growth and differentiation of the cartilage as well as the processes of perichondral osteogenesis were strikingly delayed compared to normal.

The bent, diminutive tibiae have proved a particularly suitable object for morphological analysis of the relationships between the distribution of mechanical strain and (1) the progress of differentiation of the cartilaginous model, (2) the spatial and temporal relationships between cartilage hypertrophy and perichondral osteogenesis, and (3) the structure of the periosteum and pattern of periosteal bone formation.

In the present paper the results of such analyses are reported and discussed.

Material and methods

Operations were carried out on some 120 chick embryos ranging from Hamburger-Hamilton stage 23 to stage 27. On the basis of maps of the presumptive territories of the hind-limb articles (Hampé 1959; Amprino and Camosso 1965, p. 782), the entire zeugopod, or its pre-axial half only, were isolated and proximo-distally and dorso-ventrally inverted.

The post-operative gradual changes in size and shape of the limbs were recorded by periodically inspecting the living embryos during 2–4 days. The right and left hind-limbs, isolated from specimens fixed in Bouin's fluid between 12 h and the fifth day after operation, were stained in toto with paracarmine and serially sectioned at 5 μm according to planes parallel with or perpendicular to the longitudinal axis of the zeugopod. The other embryos were fixed between the sixth and the fifteenth day following the intervention, and stained with the Lundvall method: the isolated hind-limbs, after dehydration and clearing, were photographed for study of the size and shape of the cartilaginous pieces. Both hind-limbs from some of these embryos were successively stained according to the Spalteholz method for bone tissue, and photographed again. All the operated and some control limbs were finally embedded in par-

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affin, and the entire zeugopod with adjacent parts of the stylopod and metapod were serially cut at 8 μm longitudinally or transversely for microscopical study.

Results

Periodical inspection of the living embryos revealed a speedy recovery of the continuity of the limb vascular network at both the proximal and distal levels of its interruption by isolation and rotation of the zeugopod or the preaxial part of it. An apparently normal blood circulation was reestablished within 12 to 18 h. The operated limb exhibited a lower growth rate and appeared shorter and somewhat narrower than its control, as well as misshapen at the zeugopodal level: in older embryos such deformities were often associated with reduction or failure of the spontaneous and/or passive mobility at the knee and ankle joints.

In the microscopical sections, continuity of the mesenchyme of the reoriented limb portion with that of the adjacent regions of the bud seemed reestablished within 16 to 20 h after the operation. The reoriented tissue appeared relatively denser, with few scattered mitoses: pyknotic nuclei and residues of degenerated cells were occasionally observed adjacent to the original planes of section of the inverted block till the end of the second day. Continuity of the ectoderm appeared reconstituted in buds fixed on the second day: at the planes of sealing, irregular epithelial thickenings or ridges slightly projecting on the outer surface or into the subjacent mesenchyme were often seen, especially on the dorsal aspect of buds fixed on the 2nd or 3rd postoperative day.

As to the skeletal pieces, a perceptible retardation in the initial steps of chondrogenesis of the inverted tibial blastema was observed in limbs operated upon at stages 24 or 23, i.e., two to three stages before the onset of its chondrification in the intact bud. A delay in the progress of chondrification and in the successive maturation phases of the tibia were apparent in these specimens as well as in limbs operated upon at stages 26 or 27 and fixed after 2 or 4 days (Fig. 1). Consistently, the overall size of the cartilaginous tibia was reduced in comparison to that of the control limb. In this regard, marked individual variations were recorded between embryos belonging to the same group, both in specimens operated upon at earlier or at more advanced stages. Reduction in size was associated in general with variously marked alterations in the shape of the tibia (Figs. 2, 3). This bone, always shorter and often relatively thicker than the control piece, looked sometimes like a stout cylinder with slightly expanded ends: in some of these specimens, the epiphyses were not neatly identifiable by their shape, although they could be easily recognized in histological sections on account of their parvicellular structure and the presence of vascular canals. The diminutive tibia was often variously bent or decidedly angulated at about the mid-diaphysis or, much less frequently, at the level of one metaphysis. After reorientation of the whole zeugopod the fibula did not, in general, show developmental alterations as drastic as those undergone by the tibia except in some of the specimens operated upon at stages 23 or 24 (Fig. 3f).

In the normally developing tibia, maturation of cartilage commences with cell hypertrophy at the centre of the diaphysis and gradually extends towards the extremities, so that on the 7th day in each half of the rudiment three typical zones merging into one another can be identified. This special differentiation was variously delayed by no less than 3 to 6 days in the diminutive tibiae: on the whole, retardation appeared more marked when drastic reduction in length was accompanied by a relative – sometimes also absolute – increase in thickness. Moreover, the structural differences between diaphysis, metaphysis and epiphysis were the less apparent the greater the reduction of the total mass

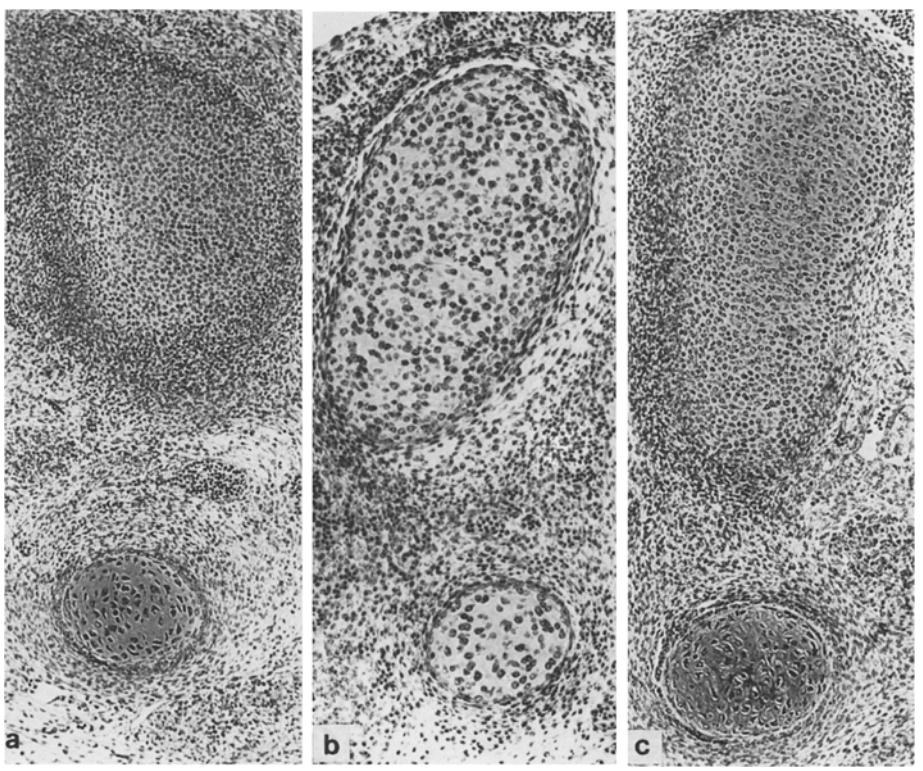

Fig. 1a–c. Cross-sections of the right foreleg. 9-day (**a**) and 10-day (**c**) embryos: proximo-distal inversion of the pre-axial half of the zeugopod at stage 25 (**a**) and 27 (**c**). ×82. **b** intact control zeugopod of a $6^1/_2$-day embryo. ×95. **a**, **c**: delayed differentiation of the inverted tibia but not of the intact fibula

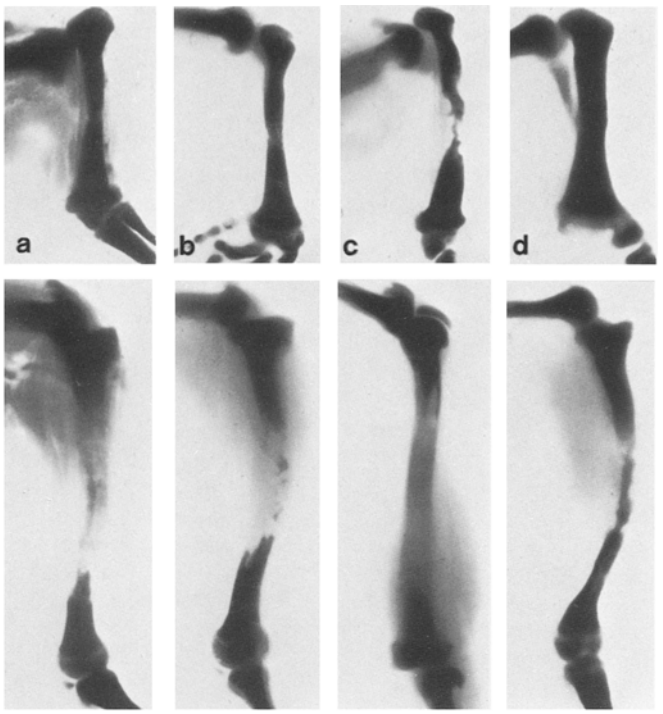

Fig. 2a–d. Moderate developmental reduction of the right tibia (*upper row*) after proximo-distal inversion of the whole zeugopod (**a**, **b**) or its pre-axial half (**c**, **d**) at stages 24 (**c**), 26 (**a**, **b**) and 27 (**d**). *Lower row*, control left tibiae

of the cartilaginous rudiment as compared to the control piece.

The onset of perichondral osteogenesis – first detectable in the normal tibia on the 7.5–8th day – was also considerably delayed and, again, relatively more in the short, stout tibiae than in the less abnormally shaped ones, i.e., those whose reduction in length was associated with an apparently proportional reduction in thickness of their various portions.

Of special interest for the analysis of cartilage maturation, perichondral bone formation, and of the temporal and spatial relationships between these two processes were the arched or angulate diminutive tibiae. The perichondrium enveloping the anterior, convex surface of the diaphysis – which in these cases was presumably subjected to longitudinal stretching greatly above normal – was dense, with thick, mostly longitudinally oriented bundles, relatively poor in cells; the distinction between an outer fibrous and a deeper, somewhat loose layer richer in cells – apparent in the normally developing contralateral piece – was barely detectable. On the opposite concave surface, the perichondrium was built of a dense outer layer consisting of a straight fibrous lamina containing numerous flattened cells scattered among parallel, thick fibre bundles. This coarse layer was anchored to the opposite epiphyses, thus subtending the bent diaphysis chord-wise. It was divided from the shaft cartilage by a space – semilunar or triangular in longitudinal section – occupied by a somewhat loose material rich in cells, corresponding to the hyperplastic inner layer of the perichondrium (Fig. 4). This space, increasingly wider in an epiphysial to mid-diaphysial direction, contained relatively thin, interlaced fibre bundles; some of these bundles appeared straight and coursed fan-like between the cartilage and the outer fibrous layer.

Unlike normal limbs (p. 50), in the bent rudiments cartilage hypertrophy set in as late as the 10th–14th day, according to the piece thickness, within a strip of the anterior convex portion lying at some distance from both the shaft central axis and the perichondrium (Fig. 4). Actually, it was separated from the latter by a band of matrix with cells growing gradually smaller and flattened the closer they be-

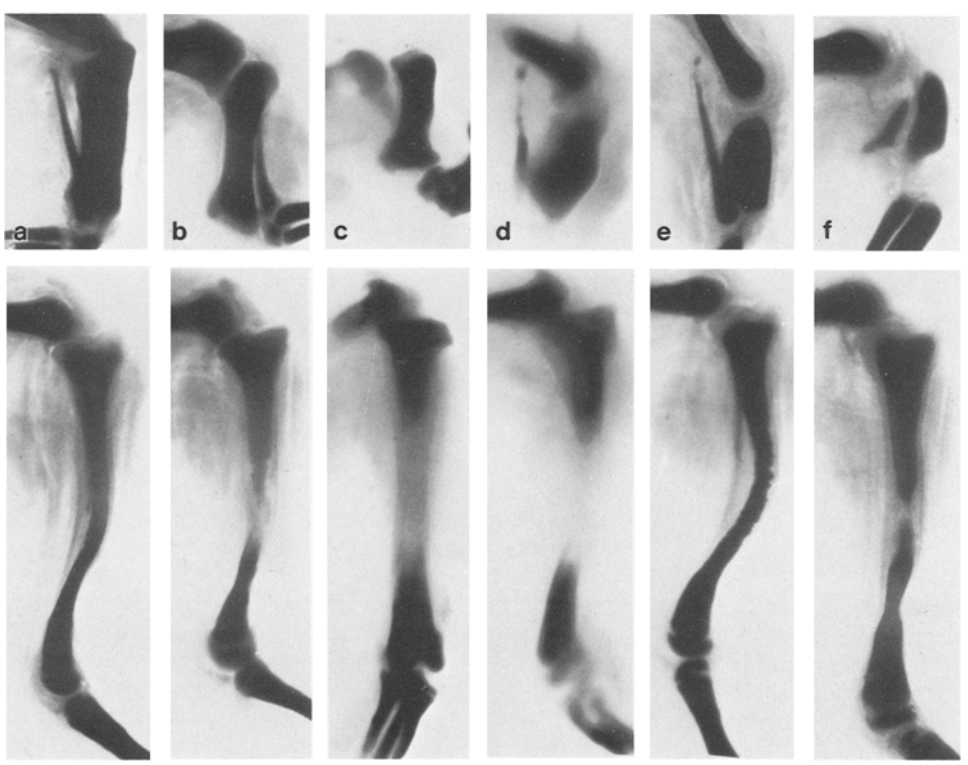

Fig. 3a–f. Alterations of varying severity in size and shape of the right tibia (*upper row*) after proximo-distal inversion of the whole zeugopod, or of its pre-axial half (**c**), at stages 23 (**f**), 25 (**a**, **b**, **c**), 26 (**d**, **e**). No marked shortening and misshaping of the pd-inverted fibula is apparent except in **f**. *Lower row*, control left tibiae

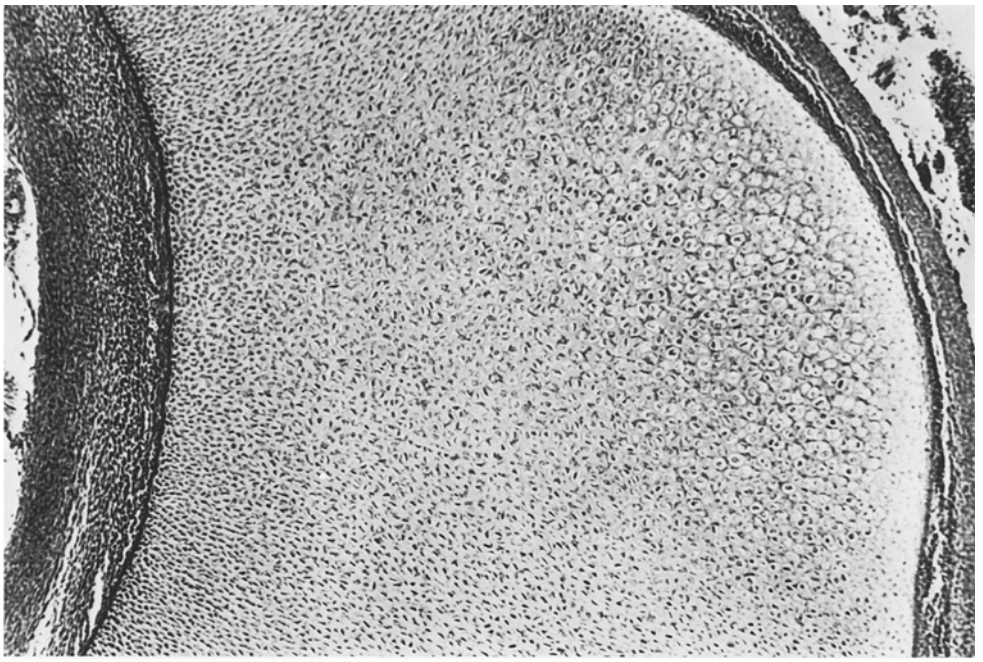

Fig. 4. 14-day embryo. Longitudinal section of the moderately bent metaphysial region of the pd-inverted tibia (stage 24). ×97. Differences in thickness and density of the perichondrium on the convex and concave aspects of the cartilage. In this tibia, cartilage hypertrophy first involved the metaphysis, i.e., the region of actual bending

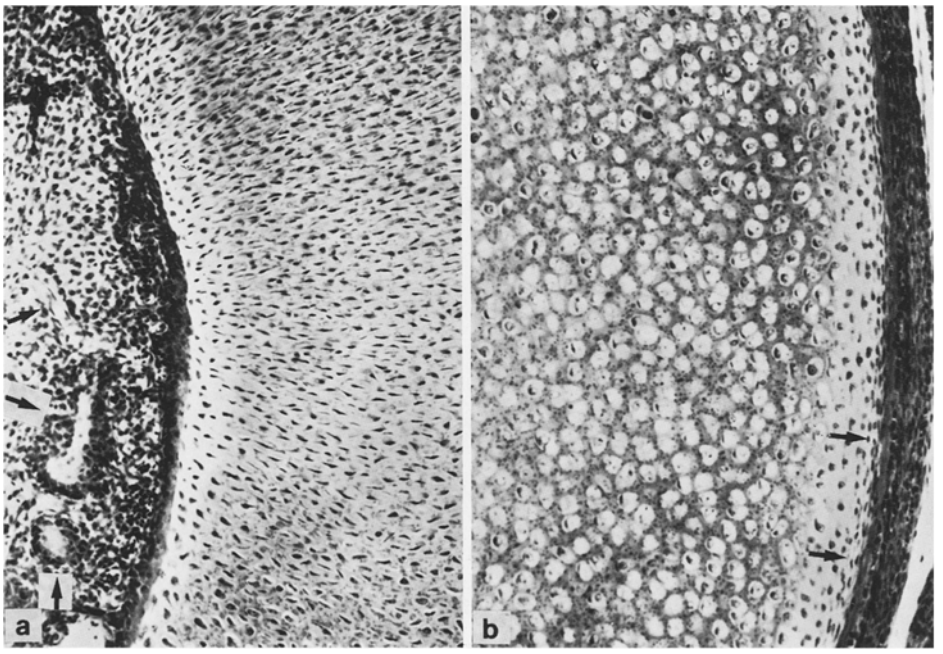

Fig. 5a, b. 14-day embryo. Longitudinal section of the bent mid-diaphysis of the pd-inverted tibia (stage $25^1/_2$). ×173. **a** in the posterior, concave region chondrocytes are small and tendentially flattened; a bone layer covers the lower half of the cartilage surface, and thin bone trabeculae (*arrows*) are forming within the richly cellular, inner layer of the perichondrium. **b** in the anterior, convex region chondrocyte hypertrophy has not attained the subperichondrial layers; a thin bone lamina is being laid down on the lower half of the cartilage surface (*arrows*)

came to the perichondrium, with their major axis oriented parallel to the surface in the subperichondral layer proper (Fig. 5b). From about the central axis to the concave surface, the diaphysial cartilage did not show morphological signs of hypertrophy: chondrocytes were small, rather flattened at places, with their major axis more or less perpendicular to the surface, a structure somehow reminiscent of that of the metaphysial zones (Fig. 5a).

As stated earlier, retardation of cartilage hypertrophy was consistently paralleled by a considerable delay in the onset of osteogenesis. Moreover, in the bent diminutive tibiae these two processes showed a variously marked incongruity with respect to each other. Often, osteogenesis commenced on the posterior, concave aspect of the mid-diaphysis within the looser, deep layer of the perichondrium which was rich in cells. A thin continuous lamina, or small, discrete spicules of bone tissue were laid down first on the surface of the cartilage, which in this region did not show morphological indications of hypertrophy (see above). Ossification then rapidly progressed along the scaffolding offered by the radially oriented collagen bundles stretched between the cartilage and the outer fibrous layer of the periosteum (p. 51). A network was formed of anastomosing bone laminae and trabeculae extending in a fan-shaped arrangement from the cartilage to the external layer of the periosteum, where they kept growing centrifugally in pace with a progressive outward shifting of the fibrous layer itself in parallel with the continuing elongation of the bent

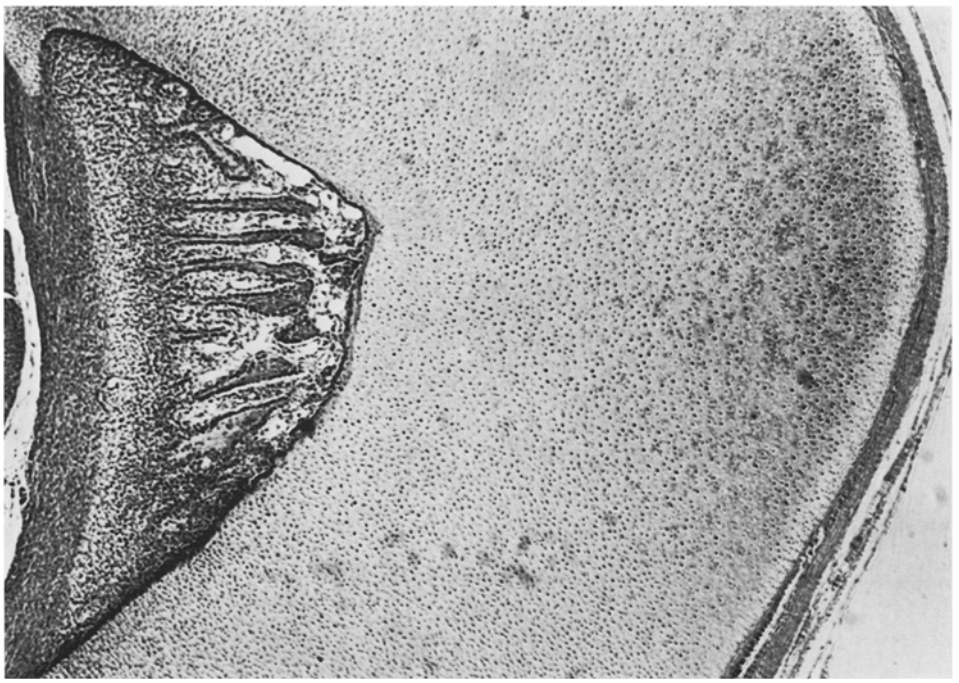

Fig. 6. 16-day embryo. Longitudinal section of the bent diaphysis of the pd-inverted tibia (stage 23). ×85. In the posterior, concave region, radially oriented bone trabeculae abut on the inner portion of the periosteum. Perichondral ossification has not yet commenced on the convex, anterior surface

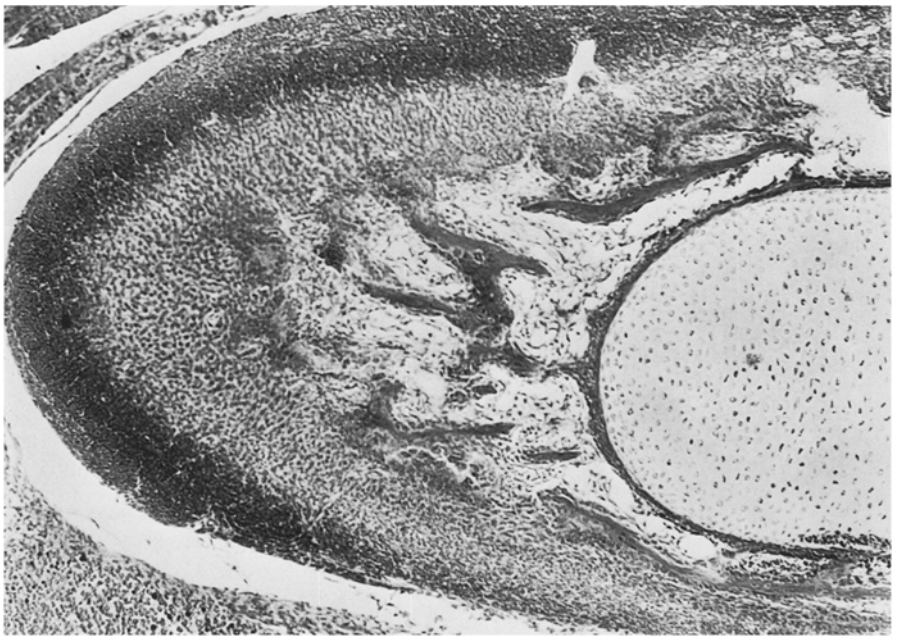

Fig. 7. 14-day embryo. Cross-section of the posterior region of the bent mid-diaphysis of the pd-inverted tibia (stage 26). ×160. A thin layer of bone envelops the cartilaginous shaft and a network of bone-trabeculae merges with the richly cellular inner portion of the periosteum

skeletal piece (Figs. 6, 7). At the same time, osteogenesis spread on both sides of the cartilaginous diaphysis towards the anterior, convex surface which was covered by a dense perichondrium relatively poor in cells. In other cases, ossification started on the convex aspect somewhat earlier than on the concave aspect of the diaphysis, where the hypertrophic changes of the cartilage were already well apparent anterior to the central axis. Not even here, however, did bone tissue form in direct contact with hypertrophic cartilage; it remained separated from the latter by a variously thick layer of parvicellular matrix (p. 52).

In specimens fixed some time – presumably four to six days – after the start of perichondral ossification, the thickness and architecture of the primary bone collar showed variations in different cases, mainly in connection with the various degree of bending of the skeletal piece. In the cross-sections of the diaphysis, the cartilaginous core – or the marrow cavity that later replaced it – consistently appeared quite eccentric, being surrounded by a ring of laminar trabecular bone whose thickness increased strikingly in an anterior to posterior gradient.

As also seen in longitudinal sections, bone was thin at the vertex of the anterior convexity, becoming gradually thicker in both longitudinal directions by formation of a series of concentric bone laminae parallel to the cartilage surface, enclosing a network of vascular channels that became narrower centripetally. When the diaphysis was markedly bent so as to form an angle of about 100–90 degrees,

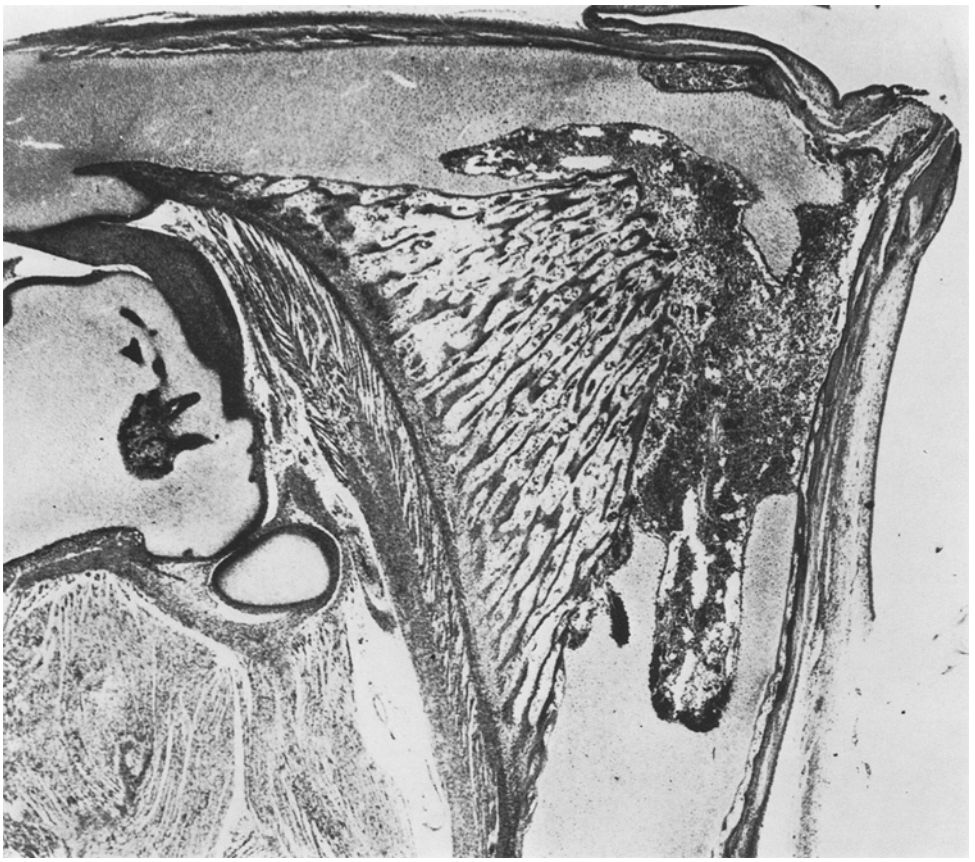

Fig. 8. 15-day embryo. Longitudinal section of the markedly angulated pd-inverted tibia (stage 26). ×45. Periosteal ossification, cartilage resorption and substitution by bone marrow are well advanced. Radially oriented trabeculae of the bone network on the concave aspect of the diaphysis. No bone has formed at the vertex of the anterior convexity where a cartilage residue is undergoing resorption

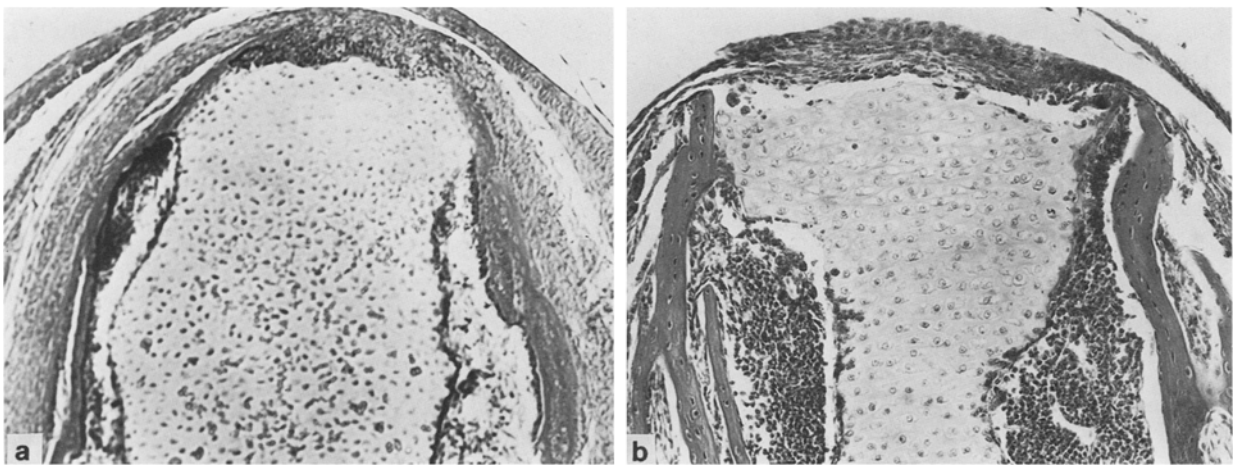

Fig. 9a, b. 14-day embryos: pd-inverted tibiae (stage 26). Anterior region of the markedly angulated mid-shaft, sectioned transversely at the vertex of the bend; **a** no perichondrial bone has been laid down in this area of the anterior surface where cartilage is undergoing resorption. ×142. **b** both the perichondrial bone previously laid down in the anterior sector and the outer layer of the subjacent cartilage have been resorbed. ×162

no bone was formed at the vertex proper (Fig. 8), but only in areas of perichondrium adjacent to it covering slightly convex or rather flat surfaces of the diaphyseal cartilage.

In general, the processes of resorption set in at the vertex of the convexity (Fig. 9) involving the bone, when present, and the subjacent cartilage where hypertrophy, first limited to a relatively deep layer (p. 52), had attained the surface.

One exceptional case, in which the pattern of perichondral bone formation on the convex side of the bent diaphysial cartilage markedly differed from that consistently observed in all the other specimens of bent tibiae, deserves special mention. Only in this sample did a prominent cap of trabecular bone – crescent-shaped in the longitudinal sections – form on top of the convexity of the diaphysial cartilage (Fig. 10a). Here, a thin and uniform layer of bone, adherent to the cartilaginous core, was continuous with an outer spongy network built of anastomosed laminae and trabeculae. In contrast to the deeper, earlier-formed layers

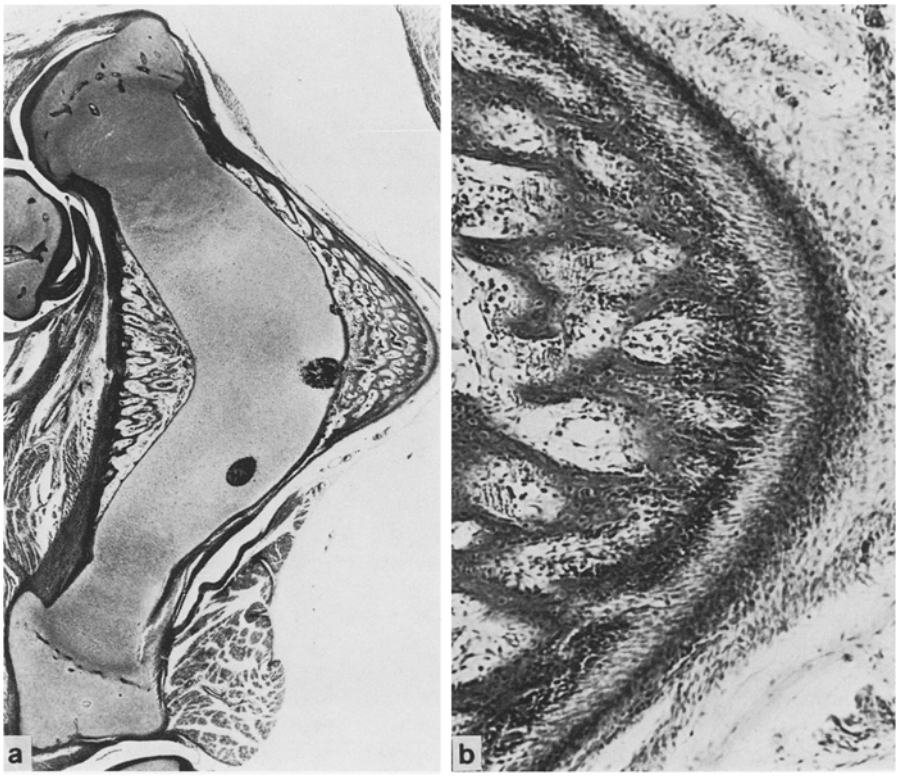

Fig. 10a, b. 15-day embryo: longitudinal section of the pd-inverted tibia (stage 25); **a** in this sample the anterior, convex aspect of the bent cartilaginous diaphysis is capped by a prominent bone outgrowth. ×21. **b** detail of the outer trabeculae growing centrifugally by incorporation of radially oriented collagen bundles of the inner periosteal layer. ×202

running parallel to the surface, these layers were arranged more perpendicularly to it, i.e., radiating toward the thick, richly cellular periosteum, whose collagen bundles – save for those of the outermost layer – appeared oriented perpendicular, not parallel, to the longitudinal bone axis (Fig. 10b). On the opposite, concave side, the arrangement of the periosteum as well as the pattern of the bony network were like those observed in the other bent tibiae.

Discussion

Reorientation of the blastema of the tibia in the chick embryo consistently hampered its development, resulting in the formation of a diminutive skeletal piece often misshapen or variously bent or angulated, whose chondrification and ossification were delayed to varying extents as compared to normal.

It should first be considered that the diminutive tibiae obtained under the present experimental conditions developed from the whole mesenchyme which gives rise to this skeletal piece in the intact, normal limb. Hence, the differences in size between experimental – showing a wide range of individual variations – and control tibiae may be a response to a variously severe reduction in growth of their early blastemata. As pointed out by Grüneberg and Lee (1973) in their study on brachypodism in the mouse, almost any disturbance of a developmental process tends to retard the growth of the structure affected: reduction in size is thus an easily detectable but unspecific concomitant of a variety of different derangements. In our material, a role in growth impairment of the experimental limbs may be tentatively ascribed to two factors at least, namely to altered local mechanical conditions and to metabolic and respiratory deficiencies. Reorientation of a part of the visco-elastic skeletogenous mesenchyme tended to bring about an increase above normal of the pressures exerted on it directly by the surrounding mesoderm of the limb bud and indirectly by the ectodermal envelope, whose expansive growth and proximo-distal sliding were – at least temporarily – arrested, thereby reducing the size of the compartment normally made available to the proliferating mesoderm. These mechanical alterations together with the temporary interruption of blood circulation may explain the greater density of the reoriented mesenchyme as well as the degeneration of a number of cells and the transient decline of cell proliferation as histologically detected in buds fixed during the first two postoperative days, especially in specimens operated upon at relatively earlier stages (pag. 50; cf. also Amprino 1974).

The reoriented mesenchyme alone was affected by the alterations of mechanical and metabolic conditions. However, in the cases of proximo-distal inversion of the whole prospective zeugopod, development of the fibula was comparatively less affected than that of the tibia. Actually, marked reduction in size associated with severe misshaping of the fibula were observed in only some of the embryos operated upon at stage 23–23$^1/_2$. This rather different behaviour of the two bone rudiments under presumably identical, or very similar local conditions might possibly be due to two main causes. Firstly, the blastema of the fibula somewhat foreruns that of the tibia in the first steps of its condensation and differentiation; therefore, at any stage between the 24th and 27th the fibular rudiment should be less deformable and compressible than that of the tibia on account of its comparatively greater physical density. Secondly, the rudiment of the fibula is thinner than that of the tibia and the cell number along the diameter of the cross sections smaller (a 42% difference at stage 25+, according to Archer et al. 1983). Hence the fibula should be subjected to a proportionally less severe reduction of the

metabolic and respiratory exchanges during the temporary postoperative arrest and the successive gradual resumption of blood circulation in the limb bud.

Despite the conceivably greater susceptibility of the undifferentiated skeletogenous mesenchyme than precartilage to the adverse early postoperative conditions, comparable growth impairment and variability in developmental deficiencies of the tibia were observed in limbs operated upon at stages 23–24 and stages 25–27: both groups exhibited a wide sequence of intermediate responses by the experimental tibiae, ranging from their drastic reduction to moderate shortening. Several aspects of long bone organogenesis could thus be analyzed in a graduated series of developmental conditions differing more or less markedly from normal.

1. Differentiation of the cartilaginous model

In the experimental limbs, differences in the onset of chondrogenesis and further development of the tibial cartilage seemed to be related to some extent to differences in thickness of the skeletal piece. A longer delay in chondrification and an even more accentuated delay in the differentiation of the cartilage into the three characteristic zones, compared to normal, were found in the short, thicker tibiae, possibly due to more significant respiratory and metabolic deficiencies. Obviously, the thicker the cartilaginous cylinder the smaller the area of the enveloping perichondrium and presumably the lower the rate of the cartilage exchanges. In the tibiae reduced in length, but not markedly altered in proportion, the hypertrophic changes at the mid-diaphysial level, though beginning later than in control bones, were less delayed.

Observations carried out on the bent, diminutive tibiae have thrown new light on the influence exerted by the mechanical strain intrinsic of the skeletal piece at the site of onset of cartilage hypertrophy and its gradual spreading to adjacent areas. Hypertrophy first involved a limited area at the more convex part of the mid-diaphysis, at some distance from the perichondrium, anterior to the central longitudinal axis: hence in a region exposed to a marked longitudinal tensile strain. Interestingly, in a few specimens where the vertex of the flexure or angulation was shifted towards one of the presumptive metaphyses, the earliest sign of cartilage hypertrophy was found to be shifted accordingly, namely to the site of presumably higher tensile strain. Signs of hypertrophy were detectable much later in the concave part of the cartilage, posterior to the central axis and thus subjected to compressive strain, the cells remaining small and, at places, flattened for a rather long period (pag. 52). It therefore seems that longitudinal tensions which presumably relieve the anterior, convex region of the bent cartilage from pressure may represent a condition favourable to cell hypertrophy – a process coupled with uptake of water by the enlarging cells – whereas axial pressures acting on the posterior, concave region of the cylinder would hinder, or at any rate further delay the onset of the hypertrophic changes. As mentioned above, in the convex region of the diaphysis hypertrophy did not initially involve the whole band of cartilage anterior to the central axis but spared for some time its outer layer subjacent to the perichondrium; this superficial band of cartilage tended to be subjected to radial compressive forces exerted upon it by the highly stretched perichondrium, and here again pressure would hinder cell swelling.

2. Relations between cartilage hypertrophy and perichondral osteogenesis

The existence of causal relationships between the onset of osteogenic activity of presumptive osteogenous cells deep-seated in the diaphysial perichondrium and the hypertrophic changes of the underlying cartilage is supported by observations on in vitro explants of long bone rudiments (Fell and Canti 1934: cf. Fell 1956). According to Fell, in normal embryonic bone osteoblasts always differentiate from the inner layer of the perichondrium as soon as chondroblastic hypertrophy begins, but not before; when hypertrophy is retarded or incomplete, no osteoblasts differentiate if the hypertrophic region is completely surrounded by parvicellular cartilage, but if in a small area it attains the surface, periosteal ossification takes place at that point only. Landauer (1933) in homozygous specimens of the *Creeper* mutant of the fowl, surviving after the fourth day, observed retardation of long bone cartilage differentiation and initial failure of zone formation linked with failure to hypertrophy: periosteal ossification was completely lacking. In connection with this finding, Fell and Landauer (1935) attempted to retard the growth of normal chick limbs in organ cultures in a growth-restricting medium; although only a small proportion of these limbs showed ossification, invariable association of ossification with hypertrophic cartilage was observed. As the growth-restricting medium did not by itself prevent bone formation, since under the same conditions membrane bone formed, Fell and Landauer concluded that suppression of perichondral bone formation in vivo or in vitro is probably a secondary effect of the retardation of growth rate which prevented cartilage hypertrophy. Some years later, Lacroix showed in the rabbit that heterotopic transplants of hypertrophic growth cartilage induce formation of a bone ring around them, which simulates the ring of the ossification groove in normal development of long bones. He postulated that the hypertrophic cartilage liberated a chemical substance ("osteogenin") which induced the differentiation of osteoblasts from the adjacent connective tissue cells (cf. Lacroix 1951). Hinchliffe and Ede (1963), in a study on chorioallantoic grafts of the shoulder girdle region from *talpid*[3] mutants of the fowl, pointed out that the membrane bone (clavicle) developed normally whereas the cartilage replacement elements (coracoid and scapula) showed no sign of cartilage replacement by bone; this non-occurrence of bone formation was assumed to be the consequence of the inability of the abnormal *talpid*[3] cartilage to induce osteogenic cells in the perichondrium.

Other authors do not uphold the existence of inductor relations between cartilage hypertrophy and osteogenesis. According to Ham (1974), the proliferating cells in the deep layer of the perichondrium begin to turn into osteoblasts as a consequence of a non-specific change in their environment, possibly brought about by the supply of more oxygen by the invading capillaries. This view, however, seems contradicted by the fact that not only formation but also resorption of perichondral bone, and of the subjacent hypertrophic cartilage, may occur in the absence of blood vessels in in vitro explants (Johnson 1980). On the other hand, Osdoby and Caplan (1979), consistent with their view that the determination of limb cell phenotypes is related to differential vascularization and nutrient flow, from experiments on in vitro cultures of chick limb bud mesenchyme

at various densities and in media of different nutritional value draw the conclusion that osteoblastic progenitor cells are present in the limb bud mesenchyme from stage 24 onwards and that initiation of bone development in the limb is not associated with cartilage development. Moreover, in the in vivo developing chick limb, the first recognizable expression of osteoblast phenotype – viz., alkaline phosphatase activity – is observed long before major cartilage core hypertrophy (Osdoby and Caplan 1981). Also according to Holder (1978), osteogenesis in the forearm of the chick embryo is programmed as from stage 24, when the first signs of chondrogenesis occur in this region. However, the onset of ossification might be controlled by some signal passing from the cartilage cells to the adjacent perichondrial cells to promote the formation of the inner osteogenic layer (cf. Lacroix 1961).

In the experimental tibiae under study, bone formation consistently started when cartilage hypertrophy was under way, but the places of perichondral osteogenesis were separated from the site of cell hypertrophy by layers, rather thick at times, of parvicellular cartilage. Although the existence of causal relationships between cartilage hypertrophy and perichondral osteogenesis – as stressed by Fell and strongly suggested by the close topographical association of these processes constantly observed in normally developing long bone rudiments in vivo and often in vitro – cannot be excluded, our material seems to indicate that direct contact of perichondrial cells with hypertrophic cartilage may not be an essential prerequisite for osteogenic induction. Diffusion of activating materials through cartilage not yet subject to hypertrophic changes may have occurred. Alternatively, as suggested by Hinchliffe and Ede (1963), it might be assumed that cartilage exerts inductor activity during chondroblast swelling but prior to a definitive hypertrophic stage. In this connection it may be recalled that investigations carried out by Stocum et al. (1979) on the biosynthetic activities of histologically distinct regions of the chick tibiotarsus from day 8 to day 18 of incubation suggest that modulation of cell biosynthetic activities in a given zone precede histological changes, since elevated chondroitin-SO_4 and collagen synthesis were recorded prior to cell flattening and hypertrophy. It could be further hypothesized that inductor influence displayed by cartilage may first involve restricted areas of the perichondrium closer to sites of initial hypertrophy, and that gradual spreading of osteogenesis to more extensive nearby areas may be due to direct influence exerted by the earliest activated osteoblasts, which might evoke osteogenic ability in surrounding perichondrial cells. A transfer of information from differentiating to undifferentiated or quiescent cells might take place through intercellular connections. EM studies suggest that the cytoplasm of adjacent bone cells are in contact, although controversy persists concerning the nature of these connections (Owen 1971; Knese 1979), i.e., whether they are "tight" or "occluding" (Holtrop and Weinger 1972) or, perhaps, "gap" junctions. Gap junctions have been documented between osteoblasts, between osteoblasts and osteocytes, and between osteocytes by several investigators (cf. Doty 1981), and it has been suggested that they may provide a means of electrical coupling between cells, establishing contact inhibition and allowing transport from cell to cell of ions and a large variety of small molecules (including metabolic intermediates, nucleotides and second messengers). The transport of small molecules has been proved by Jeansonne et al. (1979) by demonstrating that fluorescein initially injected into one cell moves through several osteoblasts. Direct cell-to-cell communication would have important implications in the formation, maintenance and destruction of bone matrix (cf. Bassett 1971). The suggestion has been advanced by several investigators that gap junctions may be assembled between contacting cell membranes but may not at all times be permeable to metabolites and ions, thus having the potential to serve as regulators in the mediation of intercellular signalling. However, gap junctions – whose presence has been shown since early developmental stages (22 to 24) between mesenchymal cells of the chick limb bud (Kelley and Fallon 1982) and between the cells of the condensing precartilaginous blastemata of the mouse embryo limb, where they persist until the onset of chondrification (Zimmermann 1984) – represent presumably only one specialized type of the various mechanisms by which cell activities might be prompted and regulated according to the conditions of their micromilieu.

3. Relations between distribution of mechanical stress and pattern of bone formation

The role played by mechanical stress in the regulation of bone formation, maintenance and reconstruction has long been debated and increasingly tested by experimental approach since the early suggestions advanced by Roux 1885 (cf. Benninghoff 1924; Weidenreich 1930; Murray 1936; Krompecher 1955; Evans 1957; Pauwels 1960, 1980; Altmann 1964; Kummer 1959, 1972; Ascenzi and Bell 1972). Of direct interest in the present context appear the in vitro studies carried out by Glucksmann (1939, 1941) on the early rudiments of long bones in chick embryos. According to this author, tensile stresses affect the development of the osseous architecture by determining where bone should be laid down in the osteogenic tissue; mechanical factors also influence the amount of bone formed in any given region, since increased normal tension of the periosteum enhances bone formation whereas tension-reduction diminishes ossification. This was shown by blocking the longitudinal expansion of unossified bone rudiments by terminal barriers, thereby provoking considerably bending: little or no bone is formed on the convex side, but an abnormally large amount of bone is laid down on the concave surface, where the perichondrium is detached from the cartilage and drawn well away from the shaft. In this space, tension lines arise which radiate outwards from the vertex of the concave surface of the cartilage: bone deposition follows these radiating tension lines. Much the same pattern of bone formation had been previously observed in vivo by Landauer (1927) in the bent limbs of chondrodistrophic chickens, by Murray and Selby (1930) and Studitski (1934) in limb bones of normal chick embryos or while developing in chorio-allantoic grafts and becoming bent.

The present observations corroborate the findings reported above. Further documentation is presented here of the influence exerted by tension or compression on bone-formation pattern in cases in which the rudiment of the tibia was not simply arched but formed an angle of about 90–100 degrees, a condition which obviously magnifies the differences in mechanical stress on the opposite surfaces of a limited segment of the cartilaginous diaphysis. In synthesis, at the vertex of the convexity the highly stretched,

outer fibrous layer of the perichondrium may exert radially oriented pressures on the inner layer and on the immediately subjacent cartilage, whose hypertrophic changes are considerably delayed (pag. 56); little or no bone is laid down here, possibly due to compression of the perichondrium inner layer which might hinder the local formation of a capillary network as well as proliferation, enlargement and transformation into osteoblasts of the deep-seated and flattened perichondrial cells. When a thin layer of bone forms in this area it is generally subject to early resorption which extends to the underlying cartilage as soon as the latter becomes hypertrophic. On the opposite, concave surface of the angulated diaphysis the outer fibrous layer of the perichondrium undergoes conspicuous thickening and is gradually drawn away from the cartilage though remaining anchored to the opposite epiphyses. In the steadily expanding area between the cartilage and the fibrous perichondrium, gradually becoming occupied by the enlarging deep layer of the perichondrium, an active cell proliferation takes place and a rich vascular network develops. These events may be favoured firstly by a decrease in the pressures that in the normally developing rudiment are exerted by the outer layer on the inner layer of the perichondrium, and subsequently by the increasing tensile forces stretching the collagen bundles which spread fan-like through this region and offer a gradually more extensive scaffolding to the prospective osteogenous cells. Perichondral osteogenesis on this concave surface may commence long before hypertrophy of the underlying cartilage becomes histologically detectable. Moreover, in this region a continuous layer of primary bone may fail to form; in some cases, in fact, discrete and regularly spaced foci of osteogenesis first appear as short bone spicules projecting from the cartilage surface at the sites of insertion on it of some of the collagen bundles stretched between the cartilage and the fibrous layer of the perichondrium. In these, much as in the more numerous specimens in which bone tissue is first laid down as an uninterrupted lamina, ossification further extends along the radial fascicles mentioned, giving rise to diverging bone trabeculae or plates which become interconnected by anastomosing osseous bridges. Due to the continued longitudinal growth of the cartilaginous model, the fibrous layer of the periosteum anchored to the epiphyses undergoes a progressive outward shift, and bone trabeculae increase in width by gradual formation of new bone at their outer extremities. This new bone abuts on the continuous, densely cellular periosteal layer which offers a further supply of cells differentiating into osteoblasts.

Only in one single case (pag. 54) out of the many subjected to histological study did a thick, prominent cap of periosteal trabecular bone form on the convex side of the bent tibia. The formation of a large amount of bone in a region where in all the other specimens osteogenesis was markedly and consistently depressed must have been prompted and sustained by local conditions peculiar to this sample. Actually, from the structure of the periosteum and the arrangement of the outer trabeculae of such bone outgrowth, it could be inferred that the latter developed under the influence of mechanical conditions substantially different from those operative in the corresponding region of all the other bent tibiae examined. The periosteum, soon after the laying down of a thin osseous layer on the surface of the diaphyseal cartilage (pag. 54), would have been submitted to radial (centrifugal) rather than longitudinal stretching so as to be gradually drawn away from the shaft, thus giving rise to tension lines which in turn determined a pattern of collagen bundle orientation and bone formation similar to that consistently observed on the concave side of the bone diaphysis. However, just what factor(s) may have exerted such a mechanical effect in the location mentioned in that single case remains a matter of speculation.

In sum, the osteogenic cells of the perichondrium and, later on, of the periosteum – which under normal conditions give rise to a primary bone collar of rather uniform thickness around the mid-diaphysis – undergo drastic local changes in number and spatial distribution as a consequence of bending or angulation of the skeletal rudiment. These conspicuous differences in the bone-forming activity of adjacent areas of the perichondrium and the periosteum seem related to local qualitative and quantitative differences in the mechanical stresses presumably affecting the recruitment and functional expression of prospective osteogenous cells. Yet the mechanisms by which stresses may influence the histogenesis of the supporting tissues are still obscure. According to Pauwels 1960; Altmann 1964; Kummer 1972, the three qualities of stresses, viz., compressive, tensile and shearing forces, cannot be considered specific stimuli of different type for the undifferentiated cell as they all cause substantially similar conditions of tension of the cell-plasma. Only stretching resulting in deformation and hydrostatic pressure should be regarded as specific stimuli: the former for the laying-down of collagen fibrils, the latter for the differentiation of cartilage, whereas no specific mechanical stimulus would play a causal role in bone tissue formation.

The experimental work on the causal relationships between mechanical stress and bone tissue formation and resorption has been considerably enhanced since it was shown that bone, cartilage and fibrous tissue may act as transducers by converting mechanical energy to electric signals. Available evidence suggests that in the supporting tissues mentioned, changes in the electric environment of the cells may control their mitotic and functional activities (cf. Bassett 1971). This is shown, e.g., by the increment of DNA synthesis of cartilage cells after a brief exposure to a pulsed electrical field (Rodan et al. 1978), and by the increase in the nuclear size of preosteoblasts preparing to enter the S phase of the mitotic cycle in response to a change in electric potential within the periodontal ligament of the rat molar subjected to loading (Roberts et al. 1981, 1982a, b). However, in spite of the expanding research (cf. Cowin et al. 1984), relatively little is known regarding the chain of events by which biomechanical stimuli may evoke response from the target cells. As to the latter, uncertainties persist on the origin of bone-forming cells in the skeleton and of cells outside the skeleton which can be induced in an osteogenic direction (cf. Owen 1982). Besides, it has not yet been ascertained whether in the developing or mature skeleton any "fibroblastic" cell, in certain sites and under some conditions of its micromilieu, can undergo modulation to become an osteoblast, or whether the prospective osteogenous cells represent a special population of early-determined osteoprogenitors (p. 56) whose proliferation and recruitment would be controlled by local conditions.

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