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The effects of paralysis on skeletal development in the chick embryo. II. Effects on histogenesis of the tibia*

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INTRODUCTION

Since Fell's classic study (1925), many workers have investigated further the histogenesis of avian long bones, especially the tibia. However, little attention has been paid to the effects of paralysis on their development. Hamburger & Waugh (1940), Drachman & Sokoloff (1966) and Bradley (1970) have all reported that the initial stages of chondrification and ossification proceed normally, but that subsequently there is a marked reduction in growth. Hall (1975) observed a 50% reduction in total growth but did not record any changes in the histological appearance of the tibia.

Our comparison paper (Hosseini & Hogg, 1991) confirmed a marked reduction in full length and in ossified diaphyseal length of the tibia but revealed no obvious differences in the time when mineralisation began in paralysed embryos. This study aimed to investigate further the qualitative and quantitative aspects of histogenesis of the tibia in paralysed chick embryos.

MATERIALS AND METHODS

Fertile eggs were incubated and embryos paralysed from 6 days of incubation onwards by treatment with decamethonium bromide solution dropped onto the chorioallantoic membrane as described in our companion paper (Hosseini & Hogg, 1991).

Histology

Four control and 4 experimental embryos were sampled at daily intervals from days 7–14 inclusive. A portion of the right limb was removed by cutting through the lower end of the femur and upper end of the metatarsus, fixed in Bouin's fluid and then transferred to 70% ethanol for 1–2 h. Specimens were then dehydrated, embedded in paraffin wax and serially sectioned transversely at a thickness of 8 μm. Three sets of a 1 in 5 series were prepared and stained with haematoxylin and eosin, alcian blue and Masson's trichrome, respectively.

Scanning electron microscopy

Similar portions of the left limbs were removed from 3 control and 3 experimental embryos at daily intervals from 12 to 16 days inclusive. These were fixed in 5% glutaraldehyde in Millonig's buffer (pH 7·4) for 24–48 h). They were supported with agar and serially sectioned transversely at 400 μm and after processing were mounted in numerical order on aluminium stubs and coated with gold. The middiaphyseal section was selected as it could be identified reliably in both groups. These were scanned with a JEOL JSMT-300 scanning electron microscope.

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Morphometry

The serial sections stained with haematoxylin and eosin from the control and experimental embryos aged 11, 12, 13 and 14 days were used for a morphometric study following the method of Gaytan, Ranz & Aceitero (1987), with the single modification that all measurements were made using a Manual Image Analysis System (Kontron MOP AM02). This method allowed the measurement and calculation of the following: total volume enclosed by the periosteum/perichondrium, volume of cartilage and its volume density, volume of invading connective and vascular tissue and its volume density, volume of the external space, i.e. osseous trabeculae and intertrabecular blood vessels and its volume density, and the volume of cartilage formed daily and volume of cartilage resorbed daily during the period investigated. Volume densities and volumes of cartilage formed daily and resorbed daily were compared (1) across the different days of incubation and (2) between control and experimental groups using a 2-way analysis of variance (F) (Statgraphics, Statistical Graphics Corporation).

RESULTS

Light microscopy

Control embryos

Early on the 6th day of incubation the cells at the centre of the shaft of the tibial condensation became elongated transversely to the long axis of the shaft and narrow intercellular spaces appeared, indicating that the formation of cartilage matrix had commenced. Towards its extremities the tissue was still composed of undifferentiated mesenchymal cells, but by the 7th day of incubation the transverse elongation of cells had spread to the ends of the diaphyseal portion of the shaft. The perichondrium was now recognisable and was bilayered around the middle region of cartilage. The cells of the outer layer were fusiform, while those of the inner layer were oval or rounded. The perichondrium was still uniform in structure around the ends of the cartilages. Some polyhedral cartilage cells in the middiaphyseal region enlarged and others showed degenerative changes. In the middle region of the diaphysis the 2 distinct layers of perichondrium were obvious and the first signs of periosteal ossification were seen, with the formation of a delicate, undulating ring composed of fine interwoven spicules.

At 8 days, 1 layer of ossified tissue surrounded the cartilage in the middiaphyseal region. At 9 days a second layer of ossified tissue had been laid down, although this was not yet a complete ring. By 10 days, 2 layers of ossified tissue had almost surrounded the middiaphyseal hypertrophic cartilage. The first layer had become thicker and many osteoblasts had been trapped in its substance, becoming osteocytes. Some radially arranged spicules connected the 2 layers of bone.

At 11 days, more bone had been deposited in the middiaphyseal region and resorption of cartilage had started after the periosteal collar of bone had been eroded by the osteogenic bud. Remnants of hypertrophied cartilage remained in the middiaphyseal region and resorption had not yet spread to the extremities of the bone.

At 12 days, there were 2–4 layers of bone in the middiaphyseal region, and these were thicker than at 11 days. Their interconnections had increased in thickness and number and the first indications of osteones were seen (Fig. 1*a*). On the lateral side of the proximal end of the tibia the fibular crest was recognisable, with its attachment to the interosseous tibiofibular ligament. Cartilage resorption had spread rapidly

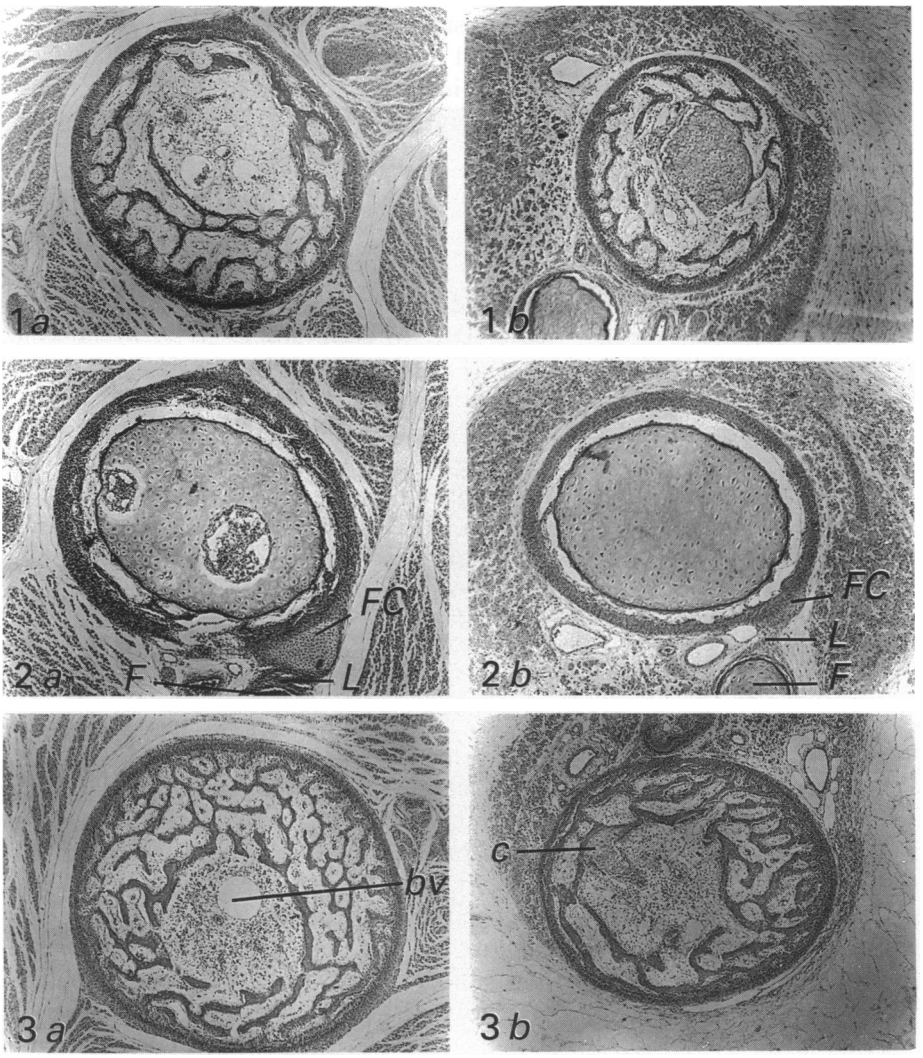

Fig. 1. Transverse sections through middiaphyseal region of tibia of control (*a*) and experimental (*b*) embryos at 12 days. In the control specimen up to 4 layers of bone are arranged in an asymmetric fashion, greater on the caudolateral aspect. Numerous interconnecting spicules are present, and the first indications of development of osteones are recognisable. All cartilage in the developing marrow cavity has been resorbed. In the experimental specimen 2 layers of bone with the initiation of a third are symmetrically arranged around the developing marrow cavity in which remnants of cartilage can be seen. Rather fewer interconnecting spicules are present. Haematoxylin and eosin. ×45.

Fig. 2. Transverse sections through proximal end of tibia of control (*a*) and experimental (*b*) embryos at 12 days. Two resorption sites are present in the control but none in the experimental specimen. In the control the fibular crest (*FC*) is better developed and can be seen attaching by a well formed tibiofibular interosseous ligament (*L*) to the fibula (*F*). Haematoxylin and eosin. ×45.

Fig. 3. Transverse sections through middiaphyseal region of the tibia of control (*a*) and experimental (*b*) embryos at 14 days. In the control specimen up to 5 layers of bones are asymmetrically arranged around the marrow cavity which contains a large blood vessel (*bv*). The number of layers and interconnecting spicules is slightly less in the experimental specimens and remnants of cartilage (*c*) persist. Haematoxylin and eosin. ×38.

towards the extremities and 2–4 absorption sites were now usually present in transverse sections of the proximal and distal ends of the tibia (Fig. 2*a*). The marrow cavity was now well formed in the central region.

At 14 days, 4–5 layers of bone with an increased number of interconnections were observed (Fig. 3*a*) and the fibular crest and interosseous tibiofibular ligament were well developed. A large central blood vessel was present within the well formed marrow cavity.

Experimental embryos

Until 11 days no differences were found in the paralysed embryos.

From 12 days onwards the tibia started to show fewer layers of bone at the middiaphyseal region than the controls (Figs 1*b*, 3*b*). Only a small fibular crest was observed with its attachment to the interosseous tibiofibular ligament.

Cartilage resorption commenced in the middiaphyseal region at 11 days, as in the controls and the osteogenic bud had progressed towards both ends of bone though not to the same extent. There were more remnants of cartilage with hypertrophic cells than in control embryos. Little change had occurred at 12 days (Fig. 1*b*). No signs of resorption were found in the extremities of the bone (Fig. 2*b*). In the middiaphyseal region the marrow cavity was well formed at 14 days, although it had not reached the ends of the bone. Remnants of hypertrophic cartilage persisted in the middiaphyseal region (Fig. 3*b*).

Scanning electron microscopy

Control embryos

At day 12, 3–5 layers of periosteal bone were seen in an asymmetric pattern, the maximum number of layers being on the caudolateral aspect of the bone. Numerous dense and compact radical spicules interconnected the circumferential layers of bone. Many blood vessels were present in the intertrabecular spaces, surrounded by loose connective tissue. The number of layers of periosteal bone gradually increased to 7 or 8 by day 16. At day 15 and 16 there were signs of resorption of the inner layer, and interconnecting spicules projected freely into the marrow cavity. The arrangement of the layers became progressively more asymmetric with the maximum number still on the caudolateral aspect (Fig. 4*a*). At day 13 compact bony trabeculae, which had formed the initial osteones, enclosed blood vessels and their surrounding connective tissue. Further bone deposition led to the development of more clearly defined osteones containing relatively smaller vessels by day 16 (Fig. 5*a*). The marrow cavity was well formed at day 12 and a large blood vessel within it was obvious from 14 days onwards.

Experimental embryos

At day 12, 2–4 layers of periosteal bone had been laid down in a fashion similar to that in the controls and numerous spicules interconnected the layers of bone. The structure of the trabeculae, the marrow cavity and developing osteones were also similar in appearance to those of the control embryos. By day 16, 5–7 layers of bone were present and there was rather less sign of resorption of the inner layers (Fig. 4*b*). The blood vessels within the central canals of the osteones were rather larger in diameter than those in the control (Fig. 5*b*) whereas those in the marrow cavity appeared rather smaller.

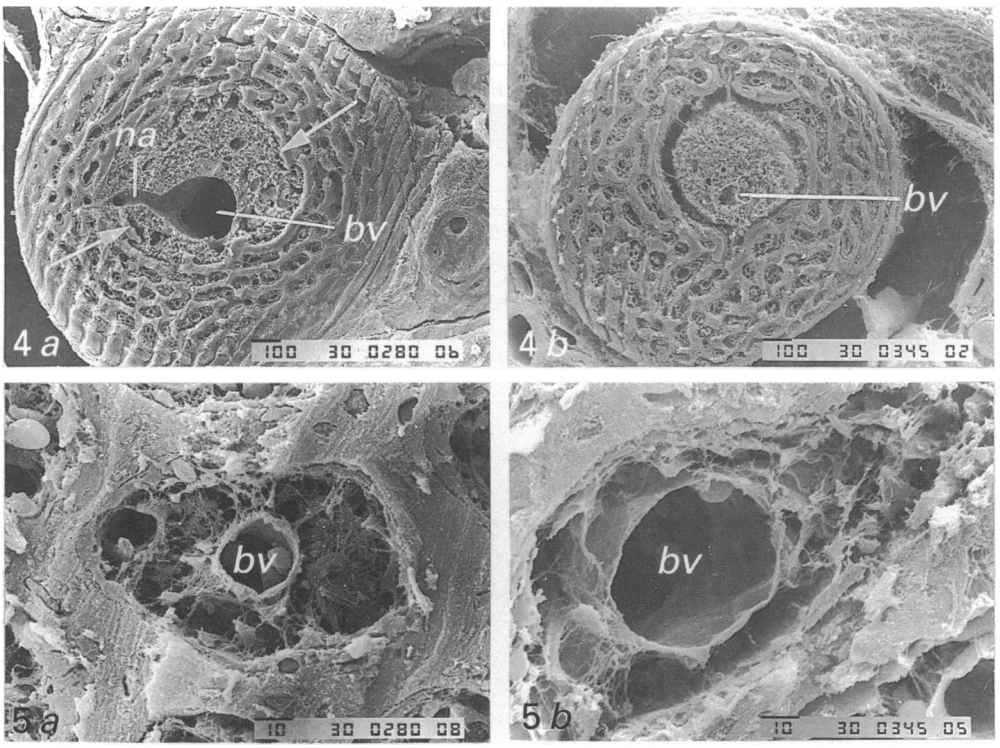

Fig. 4. Scanning electron micrographs of middiaphyseal region of the tibia of control (*a*) and experimental (*b*) embryos at 16 days. In the control specimen 7–8 layers of bone are present with numerous interconnecting spicules. Osteones are well formed as is the marrow cavity which contains a large blood vessel (*bv*) with a nutrient artery (*na*) opening into it. Remnants of the innermost layer which has been otherwise resorbed are present (arrows). In the experimental embryos the innermost layer shows little indication of resorption. Within the marrow cavity the main blood vessel (*bv*) is noticeably smaller than in the control. × 38.

Fig. 5. Scanning electron micrographs of middiaphyseal region of the tibia of control (*a*) and experimental (*b*) embryos at 16 days. In the control the blood vessel (*bv*) in the central canal is surrounded by much loose connective tissue whereas in the experimental specimen the blood vessel (*bv*) is larger and lies close to the surface of the bony trabeculae. × 500.

Morphometry

The volume enclosed by the perichondrium/periosteum and the volumes of cartilage, external space and connective and vascular tissue in the control and the experimental embryos are shown in Tables 1–4. The increases in these volumes in normal embryos have been reported previously (Gaytan *et al.* 1987). Therefore, volumes for control and experimental embryos on particular days were simply compared by Student's *t* test.

Volume enclosed by perichondrium/periosteum

Differences in total volume were apparent at 11 days, were significant at 12 days and very highly significant at 14 days.

Volume of cartilage

There were differences between the two groups which were highly significant at 14 days.

Table 1. *Volume enclosed by perichondrium/periosteum (mm^3) in control and experimental chick embryos*

Age (day)	Control mean ± SD	Experimental mean ± SD	t
11	1·9 ± 0·4	1·5 ± 0·3	1·673
12	3·1 ± 0·4	2·2 ± 0·5	2·707*
13	5·0 ± 1·0	3·1 ± 1·2	2·335
14	10·9 ± 0·9	3·3 ± 1·0	11·337***

* $P < 0·05$, *** $P < 0·001$.

Table 2. *Volume of cartilage (mm^3) in control and experimental chick embryos*

Age (day)	Control mean ± SD	Experimental mean ± SD	t
11	1·4 ± 0·3	1·1 ± 0·3	1·611
12	2·0 ± 0·4	1·4 ± 0·3	2·424
13	3·0 ± 0·7	1·9 ± 0·7	2·126
14	5·2 ± 1·2	1·9 ± 0·4	5·080**

** $P < 0·01$.

Table 3. *Volume of external space (mm^3) in control and experimental chick embryos*

Age (day)	Control mean ± SD	Experimental mean ± SD	t
11	0·5 ± 0·1	0·4 ± 0·1	1·937
12	0·8 ± 0·1	0·6 ± 0·2	2·029
13	1·5 ± 0·2	0·9 ± 0·4	2·841*
14	4·5 ± 0·8	1·0 ± 0·4	7·423***

* $P < 0·05$, *** $P < 0·001$.

Table 4. *Volume of the connective and vascular tissue (mm^3) in control and experimental chick embryos*

Age (day)	Control mean ± SD	Experimental mean ± SD	t
11	0·08 ± 0·04	0·04 ± 0·02	1·535
12	0·26 ± 0·13	0·17 ± 0·11	1·080
13	0·45 ± 0·16	0·28 ± 0·21	2·181
14	1·41 ± 0·19	0·33 ± 0·13	9·272***

*** $P < 0·001$.

Volume of external space

The smaller volume of external space in experimental embryos was significant at 13 days and very highly significant at 14 days.

Volume of connective and vascular tissue

In experimental embryos a smaller volume was obvious at 12 days and this became very highly significant at day 14.

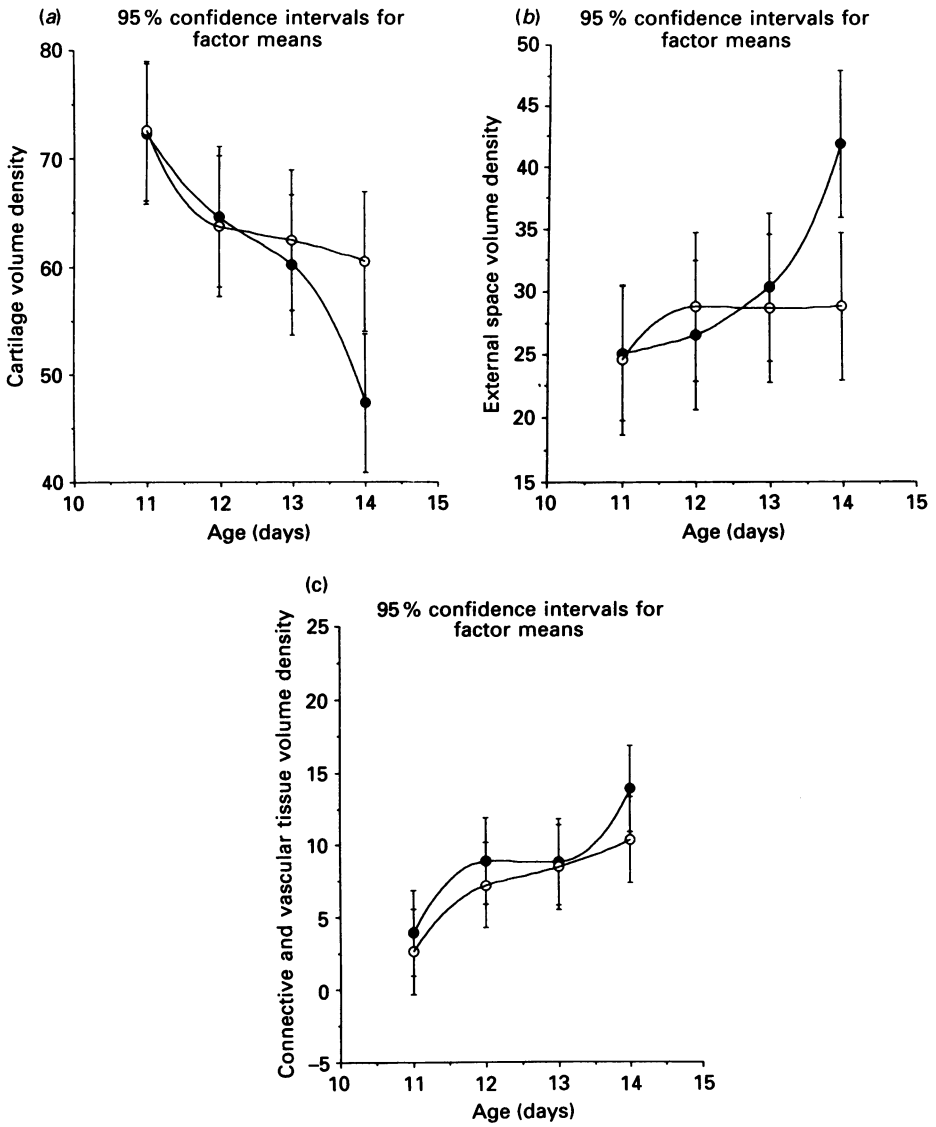

Fig. 6. Statistical comparisons of the volume densities of cartilage (*a*), external space (*b*) and connective and vascular tissue (*c*) in groups of 4 control (●) and 4 experimental (○) embryos sampled at daily intervals. The means for each group on each sample day are shown with the 95% confidence interval around each mean. Where these do not overlap groups differ significantly ($P < 0{\cdot}05$). The differences in cartilage and external space volume densities were significant at 14 days.

Volume densities of cartilage, external space and connective and vascular tissue are shown for both groups in Figure 6.

Volume density of cartilage

The cartilage volume density in both groups decreased but there was a greater decrease in control than experimental embryos. This difference was significant at 14 days.

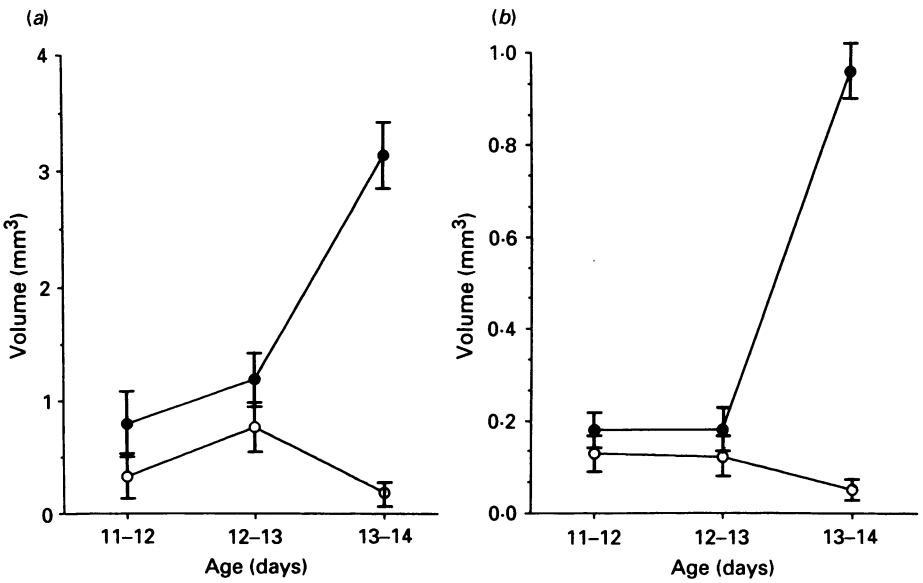

Fig. 7. Statistical comparisons of daily volumes of formation (*a*) and resorption (*b*) of cartilage in groups of 4 control (●) and 4 experimental (○) embryos. The mean values for each group for each daily period are shown with the 95% confidence interval around each mean. Where these do not overlap groups differ significantly ($P < 0{\cdot}05$). The differences in formation and in resorption were significant between 13 and 14 days.

Volume density of external space

The volume density of external space increased in both groups but not much in the experimental group, especially between day 12 and 14. The difference between control and experimental embryos was significant at 14 days.

Volume density of connective and vascular tissue

There was an increase of volume density of connective and vascular tissue in both groups but this was less in the experimental embryos. However, no significant differences were detected between the two groups.

The volume of cartilage formed daily and volume of cartilage resorbed daily in both groups are shown in Figure 7.

Formation of cartilage

Formation of cartilage decreased in the experimental embryos between days 13 and 14 when it markedly increased in the controls.

Resorption of cartilage

Resorption of cartilage markedly increased in the control embryos between days 13 and 14, whereas it occurred throughout at only very minimal levels in the experimental embryos.

DISCUSSION

The sequence of events in the control embryos for chondrification of the tibia and differentiation of the perichondrium confirms the findings of previous workers.

Specifically the onset of perichondrial ossification at 7 days (Stages 30–31) agrees with other investigations using similar histological methods (Hall, 1970; Lutfi, 1974; von der Mark, von der Mark & Gay, 1976; Scott-Savage & Hall, 1979). Similarly, the subsequent invasion of the vascular bud at the 11th day (Stages 36–37) agrees with Lutfi (1971), von der Mark *et al.* (1976), Dillaman, Wilbur & Crenshaw (1979) and Archer & Tarcliffe (1983).

All these events occurred synchronously in the paralysed embryos indicating that these events are unaffected by paralysis, at least up to this stage. From 11 days onwards, various changes started to become apparent in the paralysed embryos. The number of concentric layers of bone tissue lagged behind that in controls, suggesting a slowing in the rate of bone formation. Reduction in volume of bone present was significantly reduced in experimental embryos by 13 days. The overall form of the tibia remained generally similar to that of the controls, although the development of projections such as the fibular crest at the proximal end of the tibia was noticeably less. This is probably a direct result of lack of muscular activity, in conformity with the view of Lanyon (1980) that the general shape of bones is predetermined but that many of their dimensions and characteristic features, particularly the size and position of their crests and tuberosities, are dependent upon the presence of activity of musculature both during and after maturity.

The noticeable reduction in size of large blood vessels within the marrow cavity in the paralysed embryo may be due to the absence of muscle activity, suggested by Little (1973) as normally leading to vascular engorgement in the marrow cavity by blocking venous return. Possible variation in actual size of developing osteones and their central vessels awaits further study, but the apparently greater size of the central vessels in the paralysed chicks may again result from changes in vascular pressures during paralysis.

Subsequent to initial invasion of the vascular bud, largely unaffected by paralysis, spread of the process of cartilage resorption was identified by light microscopy as being markedly reduced by paralysis and scanning electron microscopy also suggested reduction in the normal resorption of the innermost layer of bone at 15 or 16 days. Morphometric analysis confirmed marked reduction in cartilage resorption almost to zero during the period studied. Perichondrial bone formation and cartilage formation are also markedly reduced, the latter resulting in the marked diminution in long bone length noted by previous investigators.

The mechanical force of locomotion is cyclic (Lanyon, Hampson, Goodship & Shah, 1975; Goodship, Lanyon & McFie, 1979) and is the physiological stress to which cartilage and bone respond (Hall, 1985). In the experimental embryos these mechanical forces are absent. Whether this leads directly to a lack of electrical stimuli, claimed by Currey (1968), Bassett (1971) and Hall (1975) to be the determining factor in controlling activity, or indirectly through alteration to blood supply of bone (Little, 1973) or to a lack of biochemical signals (Uchida, Yamashita, Hashimoto & Shimomuia, 1988), it is clear that the cells involved in formation and resorption both of bone and cartilage are markedly affected.

SUMMARY

In order to study the effects of paralysis on the development of bone, chick embryos were paralysed at 6 days of incubation and the pattern of histogenesis of the tibia was compared with that of control embryos by histology, scanning electron microscopy and morphometry.

Up to 11 days of incubation the histological features of chondrification, initial

perichondrial ossification and invasion by the vascular bud showed no differences. After this time the paralysed embryos exhibited a reduction in the formation of bone tissue and a reduced development of the fibular crest. The spread of cartilage resorption was also markedly reduced. In addition, scanning electron microscopy suggested a reduction in resorption of the innermost layers of bone. Blood vessels in the marrow cavity appeared smaller and those within developing osteones appeared larger than in the controls.

In the paralysed embryos, morphometry confirmed a significant reduction in total volume of the tibia together with changes in its volumetric composition resulting from reduction in bone formation, cartilage formation and cartilage resorption.

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