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The role of movement and tissue interactions in the development and growth of bone and secondary cartilage in the clavicle of the embryonic chick

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SUMMARY

There has been debate in the literature concerning whether the clavicle arises by intramembranous ossification, i.e. is a membrane bone, and whether secondary cartilage develops from its periosteal cells. A histological study of carefully staged embryos revealed that preclavicular mesenchyme undergoes condensation at H. H. stage 31–32, bone forms by H. H. stage 33 and that a transitory secondary cartilage appears late in H. H. stage 35, only to disappear by H. H. stage 36. Except for the transitory nature of the secondary cartilage, this histogenetic sequence is as seen in craniofacial membrane bones. Enzymic removal of the epithelium overlying clavicular mesenchyme from embryos of H. H. stages 26–34 and chorioallantoic grafting of the isolated mesenchyme, revealed an epithelial requirement for initiation of intramembranous ossification during H. H. stages 26–29, again similar to initiation of craniofacial osteogenesis. Secondary chondrogenesis was initiated neither in embryos paralysed with decamethonium iodide nor when clavicular mesenchyme (H. H. stages 29–33·5) was grafted to the chorioallantoic membranes of paralysed embryos, but did form in a small percentage (16–23 %) of clavicles grafted to the membranes of mobile embryos. Failure of chondrogenesis in the former was attributed to a requirement for movement as a proximate chondrogenic stimulus and the low incidence of chondrogenesis in the latter to the stimulus provided by amniotic movements which persist in paralysed embryos. Secondary cartilage did form when clavicles were organ cultured, either submerged, or at the air–medium interface. This stands in contrast to craniofacial membrane bone such as the quadratojugal, which only forms secondary cartilage *in vitro* when cultured submerged. Growth of the clavicle was shown to increase 53-fold between 10 and 11 days of incubation, an increase which was diminished but not eliminated in paralysed embryos, and which correlated closely with the dramatic increase in embryonic movement which occurs between 10 and 11 days of incubation. Thus, the clavicle of the embryonic chick shares all of the features and epigenetic requirements of the craniofacial membrane bones, but is more dependent upon biomechanical factors for its growth.

INTRODUCTION

The paired clavicles, the forerunners of the furcula or wishbone, are among the first ossifications to arise in the embryonic chick. Hamilton (1965) regarded them as the first, describing them as arising on the eighth or ninth day, a time similar to that reported by Romanoff (1960). In fact, the quadratojugal is the first bone to

Key words: clavicle, membrane bone, secondary cartilage, chick, chorioallantoic grafts, organ culture, paralysis, epithelial–mesenchymal interactions, biomechanics.

form, ossification commencing on the seventh day of incubation (Murray, 1963). Like the quadratojugal, the avian clavicle arises by intramembranous ossification, i.e. it is a membrane bone (Glenny, 1954; Bellairs & Jenkin, 1960; Romanoff, 1960; Hamilton, 1965; Ostrom, 1976). Similarly, the human clavicle develops intramembranously (Arey, 1954; Andersen, 1963; Gardner, 1968, 1971).

The classification of clavicular ossification as intramembranous is based upon studies of normal histogenesis (*ibid.*) and on the study of mutants which affect intramembranous but not endochondral ossification, viz. the *talpid* mutant in the chick (Hinchliffe & Ede, 1968) and cleidocranial dysplasia (dysostosis) in man (Jaffe, 1972; B. D. Hall, 1982). The *talpid* mutant prevents endochondral ossification from commencing by acting on the cartilaginous model. Because membrane bones of the skull and lower jaw lack a cartilaginous model, their development is unaffected by the *talpid* gene. The clavicles in *talpid* embryos behave as do the craniofacial membrane bones. Thus, when presumptive pectoral girdles are grafted to the chorioallantoic membrane, clavicles ossify normally but ossification is not initiated in the coracoid or scapula, both of which are endochondral bones (Hinchliffe & Ede, 1968). This is true, despite the fact that the clavicles and coracoid share a common embryological origin from somatopleural mesoderm (the scapula arises from somitic mesoderm, Chevallier, 1977; Chevallier, Kieny, Mauger & Sengel, 1977). Similarly, in cleidocranial dysplasia, it is the clavicles and other membrane bones which are affected – the clavicles are hypoplastic or absent, there are large fontanelles and wide sutures in the cranial vault, and wormian bones develop (Jaffe, 1972; B. D. Hall, 1982). Thus, both histogenesis and shared response identify the clavicles as membrane bones.

The above conclusions notwithstanding, identification of the clavicle as developing by intramembranous ossification has been contentious, partly because of the possible secondary fusion of cartilages to the ossifying clavicle and partly because of the possible presence of secondary cartilage on the clavicle. (Secondary cartilage is a form of cartilage which develops only on membrane bones and only after their ossification has begun (Hall, 1970, 1978*a*; Beresford, 1981).) Both the procoracoid and the hypocleideum have been identified as cartilages which fuse with the developing avian clavicle (Bellairs & Jenkins, 1960; see literature in Romanoff, 1960). The clavicles of some frogs fuse with a cartilage derived from an adjacent endochondral bone (Romer, 1960). The consensus is that the human clavicles arise as membrane bones on the ends of which secondary cartilages arise, subsequently to be replaced by bone (Arey, 1954; Gardner, 1968, 1971; Ogden, 1979). However, Andersen (1963) did not regard these cartilages as secondary; Koch (1960) regarded the human clavicle as being preformed in cartilage which then underwent premature ossification, and according to Beresford (1981) 'the first bone of the clavicle is somewhat cartilaginous' (p. 253).

The most radical reinterpretation of avian clavicular histogenesis is that carried out on the Japanese quail, *Coturnix c. japonica,* by Lansdown (1968). He described its development from an entirely cartilaginous model which was quickly invaded by osteoblasts, accompanied by calcification of the cartilaginous model, i.e. a

process of accelerated endochondral ossification. In contrasting clavicular development with that of the dentary he failed to report the secondary cartilage known to exist on the dentary (Murray, 1963). The histological technique used by Lansdown, viz. toluidine blue–silver impregnation, is not a critical technique for distinguishing cartilage from bone. Osteoid takes up toluidine blue and initial osteogenesis in avian membrane bones is characterized by large rounded cells (Murray, 1963) which could be confused with chondroblasts. The tissue identified by Lansdown (1968) as preossification cartilage (e.g. plate I.c, plate II.a,b) is, in fact, osteoid.

Lansdown's study aside, no critical search has been made for secondary cartilage on the avian clavicle, despite the fact that the presence of secondary cartilage is the norm where avian membrane bones form articulations (Hall, 1970, 1978*a*; Beresford, 1981). Such secondary cartilages are induced to differentiate from periosteal cells under the influence of mechanical forces (Hall, 1970, 1978*a*). Does the avian clavicle contain secondary cartilage and, if so, is it mechanically induced?

Ossification of membrane bones in the craniofacial skeleton is initiated as the result of an interaction between preosteogenic mesenchyme and an overlying epithelium (Hall, 1983). Is a similar epithelial–mesenchymal interaction involved in the initiation of the avian clavicle?

Past debate over the histogenesis of the avian clavicle, the paucity of studies on its day-by-day development, the total lack of experimental studies, and the implications of Lansdown's interpretation for evolutionary studies on the origin of birds (Ostrom, 1976) prompted the present study, which involves the following: (a) an analysis of the development and morphogenesis of the clavicle of the embryonic chick using histological and *in toto* skeletal staining; (b) enzymatic separation of preclavicular mesenchyme from its overlying epithelium to investigate the epithelium's role in clavicular osteogenesis; (c) grafting and organ culture of clavicles to examine secondary chondrogenesis and clavicular growth; and (d) an analysis of the growth of the clavicle, in both normal and in paralysed embryos. Specific hypotheses to be tested are detailed in the Results section.

MATERIALS AND METHODS

Incubation of eggs

Eggs of the domestic fowl (*Gallus domesticus*) were obtained from Cook's Hatchery, Truro, N.S., and incubated without turning in a humidifed incubator (Petersime Incubator No. 1, Petersime Incubator Co., Gettysburg, Ohio, USA) maintained at 37 °C.

Staging of embryos

Embryos were removed from their shells and staged using the morphological series of stages established by Hamburger & Hamilton (1951). Embryos between 5 and 16 days of incubation (H. H. stages 26–42) were used. Embryos to be used as donors for grafting or organ culture were removed from their shells and staged under asceptic conditions in a room rendered sterile by u.v. irradiation and using sterile dissecting instruments. Clavicles or clavicular mesenchyme were dissected from these embryos.

Histology

Clavicles from a series of normal embryos of H. H. stages 29–36 (6–10 days of incubation) were processed for light-microscopic histology to establish their histogenesis. Grafted or organ-cultured clavicles and clavicles from paralysed embryos were also examined histologically after fixation in neutral buffered formal–saline, dehydration in ethanol, clearing in Histoclear (National Diagnostics, Somerville, N.J., USA), embedding in paraplast plus tissue embedding medium (56°C m.p.), serial sectioning at 6 μm and staining with Mayer's haematoxylin celestine blue B, alcian blue 8G-X and direct red (Chlorantine fast red). Because this staining combination has not previously been published and because we find it to be an excellent method for the demarcation of cartilage from bone, even at the earliest stages of their differentiation, the procedure is detailed below.

Solutions

Mayer's haematoxylin (C.I. 75290) and celestine blue B (C.I. 51050) as used by Drury and Wallington (1967).

Alcian blue 8G-X (C.I. 74240). Equal parts of a 1 % aqueous solution of alcian blue and 1 % acetic acid.

Direct red (C.I. 28160): 0·5 % aqueous solution.

Phosphomolybdic acid: 1 % aqueous solution.

All stains are filtered before use.

Procedure

1. Deparaffinize slides in two changes of Histoclear, 1 min each.
2. Take slides to water in graded ethanol series (100 to 50 %).
3. Stain in celestine blue for 5 min. Rinse in distilled water.
4. Stain in Mayer's haematoxylin for 5 min. Rinse.
5. Stain in alcian blue for 5 min. Rinse.
6. Immerse in phosphomolybdic acid for 1 min. Rinse.
7. Stain in direct red for 5 min. Rinse.
8. Rapidly dehydrate in two changes of absolute ethanol, 2–3 sec/change.
9. Clear in Histoclear.
10. Mount in D.P.X.

In toto *staining of the skeleton*

To visualize the clavicles, especially their shape and size after various treatments, the skeletons of whole embryos or the clavicular region dissected from embryos were double stained with alcian blue 8G-X and alizarin red after Hanken & Wassersug (1981). This procedure stains cartilage a bright blue and bone a brilliant red and enables the very earliest sites of chondrification and deposition of calcified bone to be visualized.

Paralysis

To determine any role played by embryonic movement in either the growth of the clavicle or in the initiation of secondary chondrogenesis, embryos of H. H. stages 31–34 (7–8 days of incubation) were paralysed with a single injection of 1 mg decamethonium iodide in 0·5 ml saline. (Koch-Light Labs Ltd, Colnbrook, UK). Controls were given 0·5 ml saline. Injections were carried out using a presterilized 3 ml Tuberculin syringe fitted with a Swinnex sterilized filter unit containing a type HA, 0·22 μm porosity Millipore filter (Millipore Ltd, Montreal, Canada). The needle hole made by the injection was sealed with Scotch tape and the eggs incubated for a further 0·5–9 days, so that when examined the embryos were 8½–16 days of incubation.

This protocol for inducing paralysis of the embryonic chick was based on the method of Hall (1975). Decamethonium iodide is a postsynaptic blocking agent which acts by depolarizing the muscle membrane (Drachman, 1971). It has no deleterious actions at concentrations below those which induce paralysis. Doses above the threshold required to induce paralysis exert no additional pharmacological effect other than that caused by the paralysis (Hall, 1975 and references therein). Decamethonium is not teratogenic and therefore provides a very effective means of examining developmental events which are dependent upon embryonic movement.

At the termination of each experiment, the embryos were removed from their shells, staged, any movement noted, and the wet weight of each embryo recorded. Clavicles were then dissected from these embryos. Some were processed for histology or for *in toto* staining of the skeleton and examined for changes in histogenesis and/or morphogenesis, others were used for analysis of growth (length, wet weight) in paralysed *versus* control embryos. The resulting data were analysed using Student *t* tests and least-squares regression analysis.

Isolation of clavicular mesenchyme

To test for the possible role of an epithelial–mesenchymal interaction in the development of the membrane bone or secondary cartilage of the clavicle, preclavicular mesenchyme was separated from the overlying epithelium of the ventral body wall after enzymatic digestion. The clavicular region was dissected from embryos of H. H. stages 26–34 (5–8 days of incubation) and immersed in a 3 % solution of trypsin and pancreatin (6:1, w/w) in Ca^{2+}- and Mg^{2+}-free Tyrode's solution for 60 min at 4 °C. These tissues were then placed in a solution of Tyrode's solution and horse serum (1:1, v/v) and the epithelium dissected away from the clavicular mesenchyme using watch-maker forceps and sharpened hypodermic needles. Three types of tissues were then grown as grafts on the chorioallantoic membranes of host embryos, viz. (a) intact, non-enzyme-treated tissues, (b) intact but enzyme-treated tissues as a control for the enzyme treatment, and (c) isolated clavicular mesenchyme.

Chorioallantoic grafting

Grafts provided data on the role of epithelial–mesenchymal interactions in clavicular development. Intact or enzyme-treated clavicular tissues and isolated clavicular mesenchyme were placed onto squares of black Millipore filters (0·45 μm porosity, 125 μm thick, sterilized by soaking in 70 % ethanol and rinsing in sterile saline) and grafted to the chorioallantoic membranes of 8-day-old host embryos as described in Hall (1978*b*). Tissues were kept as grafts for 2, 3 or 8 days depending on the experiment (see Results), after which the grafts were recovered from the hosts, fixed and processed for histological or *in toto* staining of the skeleton as outlined above. In a further experiment designed to explore the role of movement in clavicular growth, clavicles were dissected from 10-day-old donor embryos, their lengths measured and then grafted onto the chorioallantoic membranes of (a) 10-day-old host embryos which had been paralysed at 9 days of incubation as described above, or (b) 10-day-old host embryos given 0·5 ml of saline at 9 days (controls). After 4 days as grafts, i.e. when the hosts had reached 14 days of incubation, the grafts were recovered, the clavicles dissected away from the adherent chorioallantoic membranes and their lengths and wet weights recorded.

Organ culture

Clavicles were asceptically removed from embryos of H. H. stages 33·75–35·75 (8–9¾ days of incubation) and established in organ culture. The clavicles were cultured submerged in 35×10 mm Falcon plastic Petri dishes, each of which contained 1·5 ml of culture medium consisting of the complex synthetic medium BGJb, supplemented with 15 % foetal calf serum (both from GIBCO Canada, Burlington, Ontario) and 225 μg of L(+)-ascorbic acid. These cultures were maintained for 3 days. The lengths of the clavicles taken from the older donor embryos were measured twice a day during this 3-day culture period. At the end of the 3 days all the cultures were fixed and processed for histological analysis.

RESULTS

Histogenesis

The normal histogenesis of the clavicle was assessed using serial sections of the preclavicular and clavicular regions of embryos of H. H. stages 29–36 (6–10 days of incubation).

There is no sign of clavicular mesenchyme at H. H. stage 29. Future clavicle-forming cells are present (Chevallier, 1977) but they have not formed a condensation and so cannot be identified. The scapula is present as a well-localized condensation, consisting of cells at the precartilaginous stage of cytodifferentiation – the cells have rounded up but not yet begun to deposit extracellular matrix.

By H. H. stage 30 ($6\frac{1}{2}$ days of incubation) chondrification of the scapula has begun (Fig. 1) and the outline of the future pectoral girdle can be seen. By H. H. stages 31–32 ($7\frac{1}{2}$ days of incubation), cartilaginous scapula, coracoid and humerus are all well differentiated (Fig. 2). The future site of the articulation between humerus and scapula can be seen as a zone of soft tissue separating the two cartilages (Fig. 2). These are also the stages when the mesenchymal condensation for the future clavicle first arises, its elongated shape presaging the shape of the clavicle (Fig. 3).

H. H. stage 33 ($7\frac{1}{2}$–8 days of incubation) marks the initiation of ossification within the preclavicular mesenchyme. The clavicle showed typical intramembranous ossification in all six specimens examined at this stage (Fig. 4) but were still mesenchymal medially where they approached one another at the ventral midline (Fig. 5). More laterally, ossification was seen as osteogenesis spread medially along the condensation (Fig. 5). By H. H. stage 34 (8 days of incubation), osteogenesis had been initiated throughout the entire preclavicular mesenchymal condensation. At its lateral tip, the clavicle lies very near a primary cartilage, from which it is always separated by a zone of unchondrified and unossified mesenchyme.

Fig. 1. Early chondrogenesis of the scapula (*s*) from an H. H. stage-30 embryo. ×122.

Fig. 2. Cartilaginous scapula (*s*), coracoid (*c*) and humerus (*h*) from an H. H. stage-31 embryo. ×28.

Fig. 3. The mesenchymal condensation of the future clavicle (arrow) at H. H. stage 31. ×28.

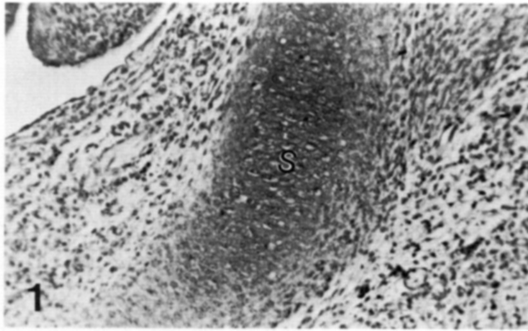
Fig. 4. Intramembranous ossification of clavicular mesenchyme at H. H. stage 33. ×122.

Fig. 5. The clavicle is mesenchymal medially (*m*) but ossifying laterally (arrow) at H. H. stage 33. ×28.

Fig. 6. Early chondrogenesis (arrow) of clavicular periosteum at H. H. stage 35. ×122.

Fig. 7. Enlarged chondrocytes (arrow) on a clavicle from an embryo of H. H. stage 35·75. ×122.

Fig. 8. Typical intramembranous bone (left) formed by H. H. stage-28 clavicular mesenchyme when grafted with the overlying epithelium for 8 days. Cartilage of the pectoral girdle is on the right. ×30.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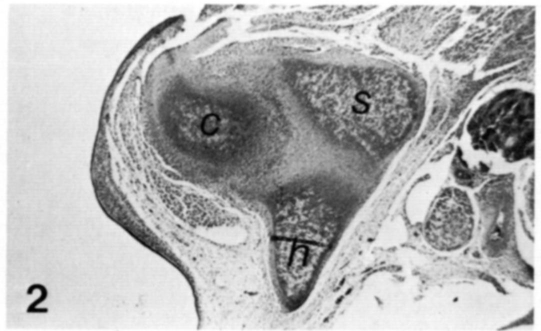
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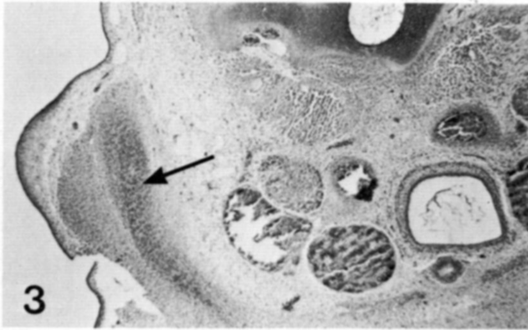
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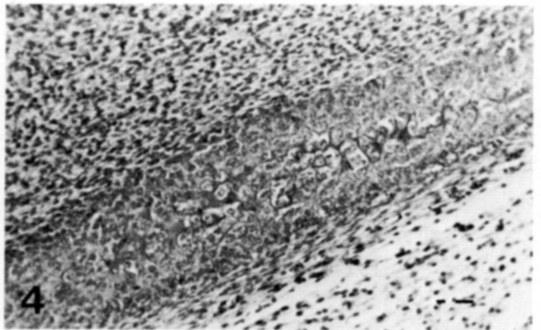
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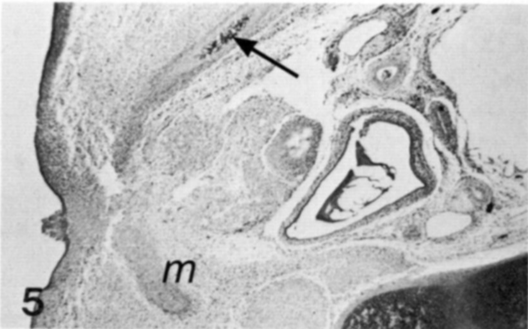
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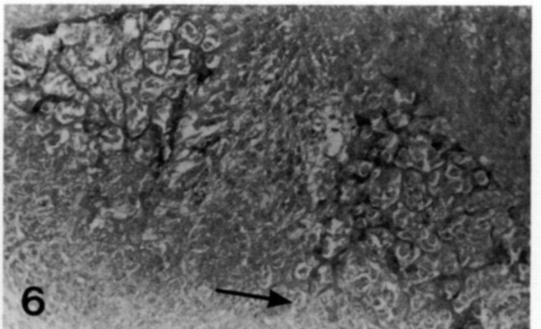
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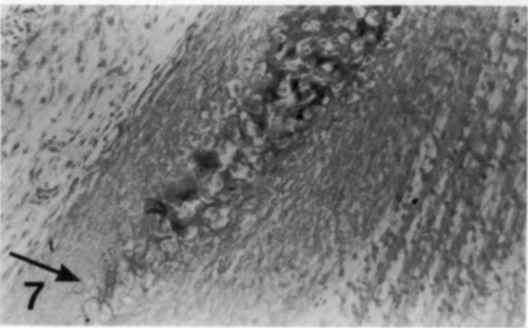
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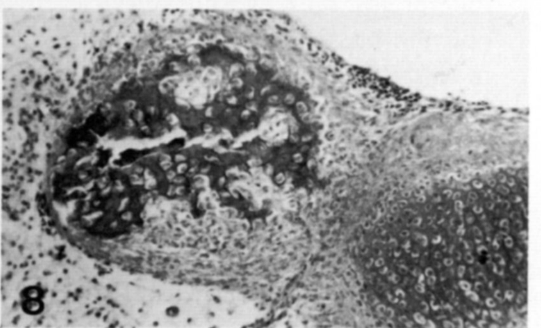
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It is at H.H. stage 35 ($8\frac{1}{2}$–9 days of incubation) that the first sign of chondrogenesis in the clavicle itself appears, first as the appearance of alcian-blue-staining extracellular matrix among periosteal cells on the lateral tip of the clavicle where it inserts onto the pectoralis muscle. This is quite distinct from the primary cartilage adjacent to the clavicle. Medially, the clavicle is characterized by considerable alcian-blue matrix staining early in H. H. stage 35.

Secondary cartilage was found in the periostea of all the six clavicles taken from embryos of late H. H. stage 35 (Fig. 6). The large chondrocytes could readily be distinguished from the adjacent very much smaller osteoblasts and osteocytes (Fig. 7). By H. H. stage 36, these chondrocytes could no longer be found.

Thus, the clavicle appears as a mesenchymal condensation at H. H. stage 31–32, intramembranous ossification begins at H. H. stage 33, alcian-blue-positive extracellular matrix appears among periosteal cells early in H. H. stage 35, and all clavicles possess secondary cartilage by late H. H. stage 35 but have lost it by H. H. stage 36.

Epithelial–mesenchymal interactions

As indicated in the Introduction, intramembranous ossification in the craniofacial skeleton is only initiated after preosteogenic mesenchyme has undergone an interaction with an adjacent epithelium, an interaction which is an absolute prerequisite for osteoblastic differentiation. The clavicle is the only membrane bone found outside the head. Would its mesenchyme also require an interaction with the overlying epithelium before osteoblasts could differentiate and bone be deposited? This possibility was tested by grafting three types of preclavicular tissue to the chorioallantoic membranes of host embryos: (1) intact mesenchyme and its overlying epithelium; (2) isolated mesenchyme, separated from the epithelium using trypsin and pancreatin; and (3) enzyme-treated but intact mesenchyme plus epithelium as a control for the enzymatic digestion. If an epithelial–mesenchymal interaction were required for bone to differentiate and if the controlled enzymic digestion had no effects on the tissues other than to destroy the basement membrane, thereby allowing epithelium and mesenchyme to be separated from one another, then bone should form in (1) and (3) above, but not in (2). This was indeed the result obtained (Table 1).

Intramembranous ossification was seen in grafts of intact mesenchyme and epithelium, whether enzyme-treated or not (Fig. 8). The number of grafts forming bone increased with donor age between H. H. stages 26 and 34 (Table 1). The first stage at which all tissues formed bone was H. H. stage 31, which is the stage when mesenchymal condensation occurs. A prominent feature in these grafts of intact tissues was the development of feathers and of cartilaginous elements of the pectoral girdle (Figs 8, 9), indicating that both the epithelium and the chondrogenic mesenchyme were unaffected by the enzyme treatment.

With the response of the intact tissues as the control we can turn to the behaviour of isolated clavicular mesenchyme. No bone was seen when the epithelium was removed before H. H. stage 29·5 (Table 1). Mesenchyme from

Table 1. *Number (per cent) of grafts of intact clavicular mesenchyme and epithelium, both non-enzyme-treated and enzyme-treated, and of isolated clavicular mesenchyme which form membrane bone after 8 days as chorioallantoic grafts*

H. H. stage (days) at extirpation	Intact (not-enzyme-treated)	Intact (enzyme-treated)	Isolated mesenchyme
26–27 (5)	3/7 (43)	4/12 (33)	0/5 (0)
28–29 (6)	4/9 (44)	4/10 (40)	0/3 (0)
29·5–30 (6½)	10/12 (83)	7/10 (70)	4/13 (31)
30–31 (7)*	8/8 (100)	10/10 (100)	7/11 (64)
33–34 (8)†	5/5 (100)	7/7 (100)	7/7 (100)

* Condensation of preclavicular mesenchyme begins at H. H. stage 31.
† Osteogenesis begins at H. H. stage 33 so that tissues isolated from embryos at or beyond that stage would all be expected to produce bone. Grafted for 2 days only.

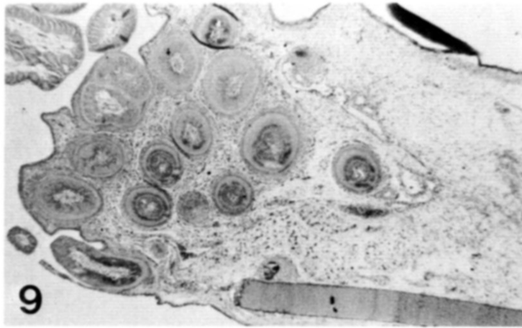
younger embryos formed muscle, primary cartilage of the medial portion of the pectoral girdle, or connective tissue. Feather development was never seen, confirming the effectiveness of the removal of the epithelium.

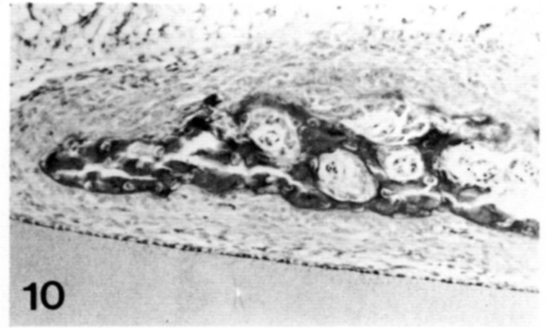
Bone formed within the isolated mesenchyme when the epithelium was removed at or after H. H. stage 29·5 (Fig. 10, Table 1), consistent with an epithelial requirement, which lasts until H. H. stage 29 and is 1½ days before clavicular mesenchyme normally condenses and 2 days before bone normally first appears.

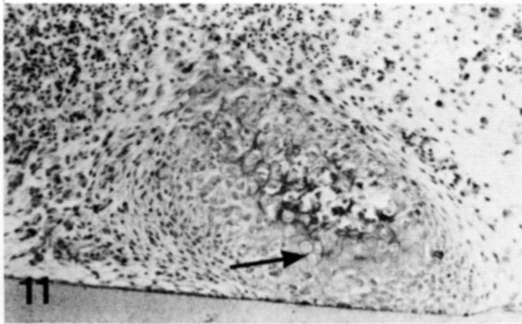
Initiation of secondary chondrogenesis

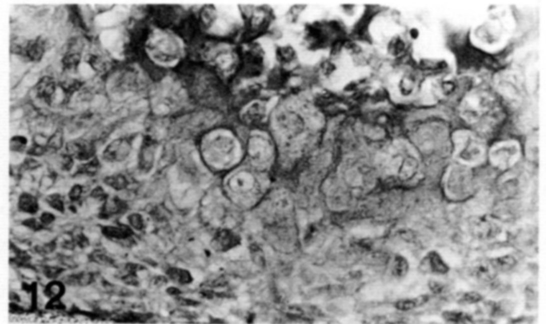
As shown in the study of normal clavicular histogenesis, alcian-blue-positive staining appears amongst periosteal cells early in H. H. stage 35, secondary cartilage appears late in H. H. stage 35 but is lost by late H. H. stage 36. What controls the initiation of secondary chondrogenesis at H. H. stage 35? As indicated in the Introduction, the other avian secondary cartilages which arise on craniofacial membrane bones are induced to form in response to biomechanical factors. To see whether this was also true for the clavicle, embryos of H. H. stages 32 and 33 were paralysed using a single *in ovo* injection of decamethonium iodide (1 mg/embryo). Examination of the clavicles at 10 days of incubation failed to reveal secondary cartilage in any of the paralysed embryos, although 60 % of the control embryos possessed secondary cartilage (Table 2). Osteogenesis was slowed in the paralysed embryos where the clavicles were thinner and often had not fused medially.

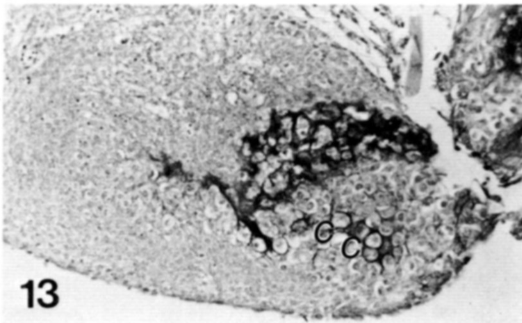
As secondary cartilage failed to form in paralysed embryos, it was predicted that it would also fail to form when clavicular mesenchyme was grafted to the chorioallantoic membranes of host embryos. However, small amounts of secondary cartilage formed in 23 % of H. H. stage 27·5–31 clavicular mesenchyme and in 16 % of H. H. stage 33–34 mesenchyme so grafted (Table 2, Figs 11, 12). Perhaps this low incidence of initiation of secondary cartilage resulted from the grafts being











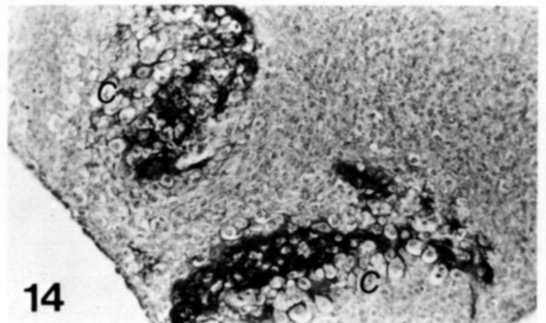


Fig. 9. Extensive development of feathers from enzyme-treated clavicular mesenchyme and overlying epithelium, isolated from an H. H. stage-30 embryo and grafted for 8 days. ×28.

Fig. 10. Typical intramembranous bone formed by H. H. stage-29·5 clavicular mesenchyme when grafted after separation from the overlying epithelium. Duration of graft life was 8 days. ×130.

Fig. 11. Secondary cartilage (arrow) around a core of membrane bone, formed when clavicular mesenchyme from an H. H. stage-33 embryo was grafted to the chorio-allantoic membrane of a host embryo for 2 days. ×130.

Fig. 12. A higher magnification photomicrograph of the secondary cartilage shown in Fig. 11. ×256.

Figs 13, 14. Secondary cartilage (*c*) formed when clavicles from H. H. stage-34 embryos were cultured submerged for 2 (Fig. 13), or for 3 (Fig. 14) days. ×130.

exposed to mechanical stimulation on the chorioallantoic membrane as the embryo moved within the shell, a possibility which was tested by grafting clavicular mesenchyme onto the chorioallantoic membranes of paralysed embryos. Embryos were paralysed at 9 days of incubation using decamethonium iodide. 24 h later, paralysis was assessed by candling the eggs and H. H. stage-29 or -33·5 clavicular mesenchyme was grafted to the membranes of paralysed embryos (Table 2). No secondary cartilage was seen in such specimens, further implicating biomechanical factors in the initiation of such cartilage.

If mechanical factors are involved then secondary cartilage should not form on clavicles or in clavicular mesenchyme maintained in organ culture, but in fact it did, in 28 % of H. H. stage-33·75 to -34 clavicles in submerged culture and in 57 % of H. H. stage-32 to -33 clavicles cultured on grids (Table 2, Figs 13, 14). Previously, avian secondary cartilage has only been shown to form *in vitro* under submerged culture conditions, and not when cultured on grids at the air–medium interface (Thorogood, 1979). This aspect will be expanded further in the Discussion.

Table 2. *Effect of paralysis, grafting or organ culturing on initiation of secondary chondrogenesis from clavicular mesenchyme*

Donor age H. H. stage (days)*	Duration of treatment (days)	No.	No. with bone	No. with secondary cartilage	% secondary cartilage†
	Embryonic paralysis				
32 (7·5)	3‡	12	12	0	0
34 (8)	2‡	12	12	0	0
	Chorioallantoic grafts				
29·5–31 (6·5–7·5)	3	44	26	6	23
33–34 (8)	3	19	19	3	16
	Chorioallantoic grafts into paralysed embryos				
29 (6–6·5)	4	5	2	0	0
33·5 (8)	3	10	10	0	0
	Organ culture (1) submerged				
33·75–34 (8)	3	19	14	4	28
	(2) on grids				
32–33 (7·5–8)	4	7	7	4	57

* See Materials and Methods for details of procedures. Samples (two to three per experiment) taken at time zero confirmed absence of secondary cartilage.

† $\frac{\text{No. with secondary cartilage}}{\text{No. with bone}} \times 100$.

‡ 60 % of clavicles from 10-day-old control embryo possessed secondary cartilage.

Growth of the clavicle

Normal embryos

The pattern of clavicular growth was assessed for normal embryos between 8 and 16 days of incubation. There is a major change in growth rate, whether measured as clavicle weight or as mg clavicle g^{-1} body weight between 10 and 11 days of incubation (Table 3). Absolute and relative clavicle weights increase 53- and 37-fold, respectively, between 10 and 11 days of incubation. Before 10 and after 11 days of incubation, clavicular growth curves have a similar slope, with the post-11-day growth curve substantially elevated (Fig. 15). Growth of the clavicle in length is much more uniform, increasing one-fold at each of 9–10, 10–11, and 11–12 days of incubation (Table 3).

The massive increase in growth of the clavicle between 10 and 11 days of incubation coincides with a substantial increase in embryonic motility, determined by comparing clavicle weight with data on embryonic motility from Hamburger, Balaban, Oppenheim & Wenger (1965). Clavicle weights of less than 5·0 mg (8–10 days of incubation) coincided with motility values of 30–55 % (Fig. 16). As soon as the average motility of the embryo exceeds 55 % (70 % at 11 days) clavicle weight shows a sharp increase (Fig. 16). The plateau in clavicle growth rate after 11 days

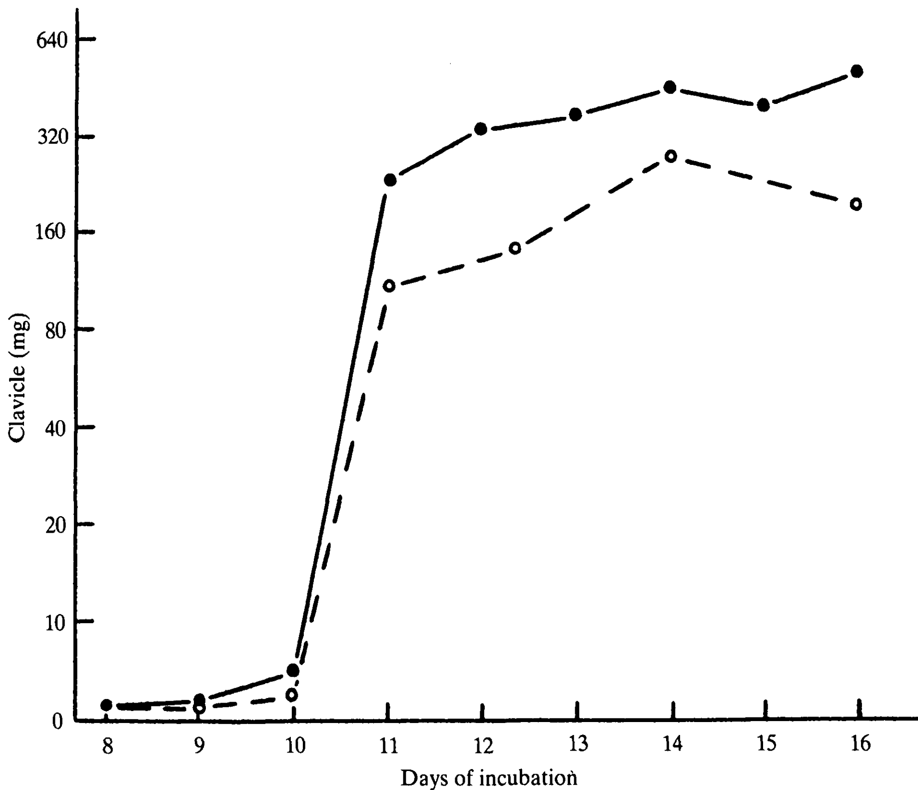


Fig. 15. Growth of the clavicle (wet weight, mg) for normal embryos (●——●) and for embryos paralysed at 7–7·5 days of incubation (○---○), over 8 to 16 days of incubation.

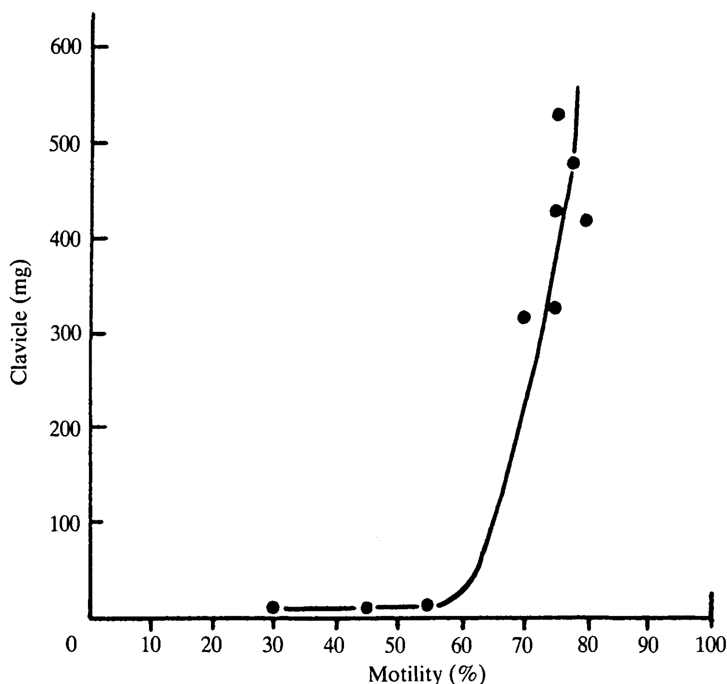


Fig. 16. Clavicle wet weight (mg) plotted against % motility for embryos of 8 to 16 days of incubation. The threshold for accelerated clavicular growth between 55 and 70 % motility corresponds to period 10 to 11 days of incubation. Data on embryonic motility from Hamburger *et al.* (1965).

of incubation parallels a levelling off in motility at around 76 % (12–16 days of incubation).

There is then a strong correlation between embryonic movement and clavicle growth, both in a threshold which stimulates a rapid increase in growth and in the subsequent slowing of clavicle growth to a rate comparable to prethreshold levels.

Paralysed embryos

(a) *Paralysis at 7–7·5 days.* If the correlation of clavicular growth with movement is a causal one, then elimination of embryonic movement should either prevent the clavicle from growing at all (if all its growth is dependent upon mechanical stimulation) or slow its growth (in proportion to the dependence on movement). This hypothesis was tested by paralysing embryos at 7–7·5 days of incubation (the age when the clavicular mesenchyme condenses), and examining subsequent growth of the clavicle to 16 days of incubation (Table 3).

Paralysis suppressed general body growth so that by 14 days of incubation paralysed embryos were significantly smaller than control embryos. Between 8 and 16 days of incubation some 21 % of body growth was dependent on embryonic movement.

Growth of the clavicle was much more dependent on movement than was general body growth, for absolute and relative clavicle weights were significantly

smaller in paralysed embryos from 9 days of incubation onwards (Table 3, Fig. 15). Clavicle length was significantly smaller from 10 days onwards. Between 8 and 16 days of incubation, 52 % of absolute and 41 % of relative clavicle growth failed to occur in paralysed embryos. Fourteen per cent of increase in length was movement-dependent between 9 and 16 days. Regression equations ($Y = mx+c$) were calculated for clavicle length (x) versus weight (Y) for both normal and paralysed embryos over 9–16 days of incubation and took the form: control, $Y = 155{\cdot}22x - 489{\cdot}30$; paralysed, $Y = 88{\cdot}34x - 240{\cdot}97$.

These data therefore show that some 40 % of relative clavicle growth in mass and 14 % of increase in length is dependent on embryonic movement.

(b) *Paralysis at 10–11 days.* The relationship between embryonic movement and clavicular growth illustrated in Fig. 16 suggested that the clavicle might be responding in a threshold way to a single increase in embryonic motility between 10 and 11 days, rather than responding continuously to increased motility from 10 days onwards. To test this concept, embryos were paralysed during (10·7 days) or just after (11·7 days) the increase in clavicle growth shown to occur between 10 and 11 days (Fig. 15), and clavicle growth compared with controls. Paralysis at both ages resulted in reduced clavicular growth in comparison to control (Table 4)

Table 3. *Body weights (g), clavicle weights (mg) and lengths (mm) for control and paralysed embryos**

Age	Body weight (g)	Clavicle weight (mg)	Clavicle ($mg\,g^{-1}$ body weight)	Clavicle length (mm)	No.
			Control		
8	1·10 ± 0·03	1·50 ± 0·09	1·36	—	10
9	1·53 ± 0·03	2·16 ± 0·15	1·41	3·4 ± 0·05	16
10	2·46 ± 0·07	4·85 ± 0·33	1·98	3·81 ± 0·04	9
11	3·47 ± 0·14	259·3 ± 21·9	74·10	4·35 ± 0·07	11
12	4·48 ± 0·14	327·4 ± 13·2	73·2	4·69 ± 0·08	15
13	7·81 ± 0·22	414·0 ± 56·4	53·00	5·67 ± 0·07	10
14	9·00 ± 0·16	483·2 ± 29·4	53·68	6·14 ± 0·07	23
15	12·97 ± 0·29	422·4 ± 26·7	33·0	6·43 ± 0·08	10
16	14·04 ± 0·39	532·8 ± 36·2	38·48	6·65 ± 0·06	8
			Paralysed†		
8	1·11 ± 0·07	1·73 ± 0·12	1·56	—	14
9	1·62 ± 0·04	1·76 ± 0·05§	1·09	3·0 ± 0·04	22
10	2·13 ± 0·08	2·97 ± 0·18‡	1·39	3·37 ± 0·06‡	18
11	2·64 ± 0·12§	115·1 ± 7·9‡	44·6	3·72 ± 0·05‡	11
12	4·16 ± 0·11	147·0 ± 4·8‡	35·3	4·35 ± 0·05‡	22
14	7·70 ± 0·27§	287·5 ± 13·8‡	37·3	4·73 ± 0·06‡	22
16	9·40 ± 0·52‡	205·0 ± 15·3‡	21·80	5·69 ± 0·13‡	11

* Values are $\bar{x} \pm$ S.E.M.
† Embryos paralysed at 7–7·5 days of incubation.
‡ Paralysed significantly smaller than control at $P > 0{\cdot}001$.
§ Paralysed significantly smaller than control at $P = 0{\cdot}01–0{\cdot}005$.

indicating that significant clavicular dependence on movement extends beyond 11 days of incubation. Thus, dependence of clavicular growth on movement extends beyond the threshold response at 10–11 days.

(c) *Grafts into paralysed embryos*. The histological analysis of clavicles and clavicular mesenchyme grafted to the chorioallantoic membrane indicated some increase in osteogenesis (growth) during time as a graft. Given that much clavicular growth is dependent upon movement and that the chorioallantoic membrane is subject to movement from both embryonic motility and from rhythmic contraction of the amnion, the growth on the chorioallantoic membrane may have been in response to mechanical stimulation from such movement. This was tested by grafting clavicles from 10-day-old embryos to the chorioallantoic membranes, either of control embryos, or of embryos paralysed at 9 days of incubation. The length of each clavicle was measured before grafting and after 4 days as a graft. Clavicles grafted to mobile embryos increased in length by 12 %, clavicles grafted to paralysed embryos failed to grow (Table 5). There was much more variation in paralysed than in control hosts. All grafts to control embryos increased in length whereas four out of seven grafts to paralysed embryos decreased in length, while three showed some growth (Table 5). Normally, the clavicle would increase in length by 61 % between 10 and 14 days of incubation (Table 3). Therefore, any growth of grafted clavicles was in response to mechanical stimulation experienced on the chorioallantoic membrane.

DISCUSSION

The histological analysis of clavicular development indicated that the clavicle of the domestic fowl is a typical membrane bone. It appears as a condensation of mesenchyme at H. H. stages 31–32, the shape of which outlines the linear

Table 4. *Effect of later paralysis on clavicle growth* ($\bar{x} \pm$ S.E.M.)

Age at paralysis in days (H. H. stage)	Body weight (g)	Clavicle weight (mg)	Clavicle (mg g^{-1} body weight)	Clavicle length (mm)	No.
10·7 (36·5)					
control*	9·29 ± 0·36	332·0 ± 27·7	35·16	5·70 ± 0·09	15
paralysed	8·12 ± 0·58	243·0 ± 26·7‡[1]	29·61	4·90 ± 0·17‡[2]	8
P/C %	87 %	73 %	84 %	86 %	
11·7 (37·5)					
control†	4·45 ± 0·14	284·5 ± 19·6	63·8	4·77 ± 0·13	6
paralysed	4·66 ± 0·22	190·2 ± 11·5‡[3]	41·3	4·37 ± 0·09‡[4]	5
P/C %	105 %	67 %	65 %	92 %	

* Examined at 14 days of incubation.
† Examined at 12 days of incubation.
‡ Significantly smaller than control at $P = 0{\cdot}02–0{\cdot}01$ (‡[1]); 0·005–0·001 (‡[2]); 0·01–0·005 (‡[3]); 0·10–0·05 (‡[4]).

morphology of the future clavicle. Intramembranous ossification rapidly follows at H. H. stage 33, just 12 to 18 h after condensation. It begins laterally and by H. H. stage 34, all of the condensation is ossified. There was no evidence of a cartilage model as described by Lansdown (1968) for the Japanese quail (*Coturnix c. japonica*). Very recently, Russell & Joffe (1985) have reanalysed the development of the clavicle in the Japanese quail and confirm its development by intramembranous ossification.

Only *after* osteogenesis had been initiated along the whole length of the mesenchymal condensation (which occurs by H. H. stage 34) did the first signs of chondrogenesis appear as alcian-blue-staining extracellular matrix between periosteal cells on the lateral tips of each clavicle at H. H. stage 35, some 24 h after initiation of osteogenesis at that site. Some 12 h later, late in H. H. stage 35, secondary cartilage had differentiated in all specimens. 12 h later, at H. H. stage 36, the cartilage could no longer be seen. Such a rapid disappearance of the

Table 5. *Effect of paralysis of host embryos on growth of grafted clavicles*

	Clavicle length (mm)		
Number	Pregraft*	After 4 days†	Change
	Grafts into mobile embryos		
1	3·98	4·38	+0·40
2	3·74	4·15	+0·41
3	3·57	4·38	+0·81
4	3·86	4·38	+0·52
5	3·92	4·38	+0·46
6	3·62	4·03	+0·41
7	3·92	4·33	+0·41
8	3·74	3·92	+0·18
$\bar{x} \pm$ S.E.M.	3·79 ± 0·05	4·24 ± 0·06	0·45 mm
% increase		11·9 %	
	Grafts into paralysed embryos		
1	3·86	3·51	−0·35
2	3·92	3·74	−0·18
3	4·27	3·92	−0·35
4	3·80	4·44	+0·64
5	4·09	4·27	+0·18
6	3·62	3·74	+0·12
7	3·86	3·80	−0·06
$\bar{x} \pm$ S.E.M.	3·92 ± 0·07	3·92 ± 0·11	0 mm
% increase		0 %	
P‡	NS	0·05 = 0·025	

* As measured when clavicle dissected from 10-day-old donor embryos.
† As measured when clavicle recovered from 14-day-old host embryo after 4 days as chorio-allantoic graft.
‡ Comparison of mobile with paralysed.

secondary cartilage stands in marked contrast to the fate of similar cartilages on craniofacial membrane bones, where most of the cartilage persists into adult life, only then to be replaced by bone or by a fibrous articular cartilage or to be transformed into a fibrocartilage (Hall, 1967, 1968). Similarly, secondary cartilages in the facial skeleton of the rat vary very considerably both in the time at which they arise and in their lifespan (Vinkka, 1982).

The initiation of osteogenesis in clavicular mesenchyme was shown to be dependent upon the overlying epithelium being present until H. H. stage 29, i.e. until $1\frac{1}{2}$ days before osteogenesis commences at H. H. stage 33. This is a shorter interval between the end of the epithelial requirement and initiation of osteogenesis than has been described for craniofacial membrane bones, where an interval of 3 days is more typical (Tyler & Hall, 1977; Hall, 1983). All avian membrane and dermal bones which have been examined require such an epithelial–mesenchymal interaction before osteogenesis can be initiated (Hall, 1983, 1984).

The secondary cartilage which appeared late in H. H. stage 35 was shown to be dependent upon embryonic movement for its formation: it failed to form in paralysed embryos or when clavicles were grafted to paralysed embryos, but did form in some clavicles maintained in organ culture. The secondary cartilages which arise on craniofacial membrane bones (surangular, squamosal, quadratojugal) appear late in H. H. stage 36 ($10\frac{3}{4}$ days of incubation), a time which coincides with a rapid increase in embryonic motility; embryos are active during 55 % and 70 % of the time at 10 and 11 days of incubation, respectively (Hamburger *et al.* 1965). Clavicular secondary cartilages arise at a time when embryonic motility is changing from 45 % of the time (H. H. stage 35) to 55 % of the time (H. H. stage 36). It thus appears either (a) that some factor other than, and independent of, movement is required for initiation of clavicular secondary cartilage, or (b) that the threshold of movement is lower for the clavicle than for the craniofacial membrane bones. The complete absence of secondary cartilage in paralysed embryos (Table 2) eliminates (a). The fact that quadratojugals grafted into paralysed embryos fail to form secondary cartilage (unpublished observations) and that clavicles form secondary cartilage under *in vitro* conditions (culture at the air–medium interface) which are not sufficient to allow craniofacial membrane bones to form secondary cartilage (Thorogood, 1979), are both also consistent with the clavicle having a lower threshold to mechanical initiation of cartilage. Embryonic motility acts by selective enhancement of the proliferation of periosteal cells (Hall, 1979). The link with *in vitro* secondary chondrogenesis is that submerged organ culture also stimulates proliferation.

Clavicular growth was also shown to be rapidly accelerated in parallel with increasing embryonic motility between 10 and 11 days of incubation. This relationship of clavicular growth to embryonic motility was shown by (a) the correlation with motility, (b) the significant slowing of growth with paralysis, and (c) the absence of growth when clavicles were grafted to the chorioallantoic membranes of paralysed host embryos. Such a correlation is not typical of the

growth of craniofacial membrane bones, which form extensive and long-lasting secondary cartilage over the same period, and whose growth is not dramatically slowed by paralysis (Hall, 1979). Thus the periosteal cells of craniofacial membrane bones are specialized to respond to a threshold of embryonic motility (55–70 % activity) by forming extensive secondary cartilage between 10 and 11 days of incubation. The clavicle responds to a lower threshold of motility (45–55 % motility) between 9 and 10 days of incubation by forming secondary cartilage. With the increasing motility between 10 and 11 days of incubation, periosteal clavicular cells switch to extensive osteogenesis and away from chondrogenesis. There is thus both similarity and interesting differences between these two different membrane bones. The mammalian clavicle shows similar responses to mechanical factors in that compression at the site of a fracture in the human clavicle initiates chondrogenesis and formation of a pseudoarthrosis (Koskinen-Moffett & Moffett, 1984); induced bipedalism in rats increases mechanical stimulation to the clavicles thereby stimulating their growth (Riesenfeld, 1966), and the muscular dysgenesis (*mdg*) mutation in the mouse results in a deficiency of all the skeletal muscles and consequent shorter than normal clavicles (Pai, 1965).

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REFERENCES

ANDERSEN, H. (1963). Histochemistry and development of the human shoulder and acromioclavicular joints with particular reference to the early development of the clavicle. *Acta Anat.* **55**, 124–165.

AREY, L. B. (1954). Developmental Anatomy. *A Textbook and Laboratory Manual of Embryology.* Philadelphia, Pa.: W. B. Saunders Inc.

BELLAIRS, A.D'A. & JENKIN, C. R. (1960). The skeleton of birds. In *Biology and Comparative Physiology of Birds*, vol. **1** (ed. A. J. Marshall), pp. 241–300. New York: Academic Press.

BERESFORD, W. A. (1981). *Chondroid Bone, Secondary Cartilage and Metaplasia.* Munich, Baltimore: Urban and Schwarzenberg.

CHEVALLIER, A. (1977). Origine des ceintures scapulaires et pelviennes chez l'embryon d'oiseau. *J. Embryol. exp. Morph.* **42**, 275–292.

CHEVALLIER, A., KIENY, M., MAUGER, A. & SENGEL, P. (1977). Developmental fate of somitic mesoderm in the chick embryo. In *Vertebrate Limb and Somite Morphogenesis* (ed. D. A. Ede, O. P. Flint, O. K. Wilby & P. Colquhoun), pp. 421–432. Cambridge: Cambridge University Press.

DRACHMAN, D. B. (1971). Neuromuscular transmission of trophic effects. *Ann. N.Y. Acad. Sci.* **183**, 158–170.

DRURY, R. A. B. & WALLINGTON, E. A. (1967). *Carleton's Histological Technique.* New York: Oxford University Press.

GARDNER, E. (1968). The embryology of the clavicle. *Clin. Orthop. & Related Research* **58**, 9–16.

GARDNER, E. (1971). Osteogenesis in the human embryo and fetus. In *The Biochemistry and Physiology of Bone*, vol. **3** (ed. G. H. Bourne), pp. 77–118. New York: Academic Press.

GLENNY, F. H. (1954). The clavicles and dorsal coracoid arteries as indices of phyotic relationships and levels of avian evolution. *Anat. Anz.* **101**, 95–100.

HALL, B. D. (1982). Syndromes and situations associated with congenital clavicular hypoplasia or agenesis. In *Skeletal Dysplasia* (ed. C. J. Papadatos and C. S. Bartsocas), pp. 279–288. New York: Alan R. Liss, Inc.

HALL, B. K. (1967). The distribution and fate of adventitious cartilage in the skull of the eastern rosella, *Platycercus eximius* (Aves: Psittaciformes). *Aust. J. Zool.* **15**, 685–698.

HALL, B. K. (1968). The fate of adventitious and embryonic articular cartilage in the skull of the common fowl, *Gallus domesticus* (Aves: Phasianidae). *Aust. J. Zool.* **16**, 795–806.

HALL, B. K. (1970). Cellular differentiation in skeletal tissues. *Biol. Rev. Camb. Phil. Soc.* **45**, 455–484.

HALL, B. K. (1975). A simple, single-injection method for inducing long-term paralysis in embryonic chicks, and preliminary observations on growth of the tibia. *Anat. Rec.* **181**, 767–778.

HALL, B. K. (1978*a*). *Developmental and Cellular Skeletal Biology.* New York: Academic Press.

HALL, B. K. (1978*b*). Grafting organs and tissues to the chorioallantoic membrane of the embryonic chick. *Tissue Culture Assoc. Manual* **4**, 881–884.

HALL, B. K. (1979). Selective proliferation and accumulation of chondroprogenitor cells as the mode of action of biomechanical factors during secondary chondrogenesis. *Teratology* **20**, 81–92.

HALL, B. K. (1983). Epithelial–mesenchymal interactions in cartilage and bone development. In *Epithelial–Mesenchymal Interactions in Development* (ed. R. H. Sawyer & J. F. Fallon), pp. 189–214. New York: Praeger Press.

HALL, B. K. (1984). Matrices control the differentiation of cartilage and bone. In *Matrices and Cell Differentiation* (ed. R. B. Kemp & J. R. Hinchliffe), pp. 147–170. New York: Alan R. Liss, Inc.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in development of the chick embryo. *J. Morph.* **88**, 49–92.

HAMBURGER, V., BALABAN, M. OPPENHEIM, R. & WENGER, E. (1965). Periodic motility of normal and spinal chick embryos between 8 and 17 days of incubation. *J. exp. Zool.* **159**, 1–14.

HAMILTON, H. L. (1965). *Lillie's Development of the Chick.* New York: Holt, Rinehart and Winston.

HANKEN, J. & WASSERSUG, R. (1981). The visible skeleton. *Functional Photography* **16**, 22–26.

HINCHLIFFE, J. R. & EDE, D. A. (1968). Abnormalities in bone and cartilage development in the *talpid*^{3} mutant of the fowl. *J. Embryol. exp. Morph.* **19**, 327–339.

JAFFE, H. L. (1972). *Metabolic Degenerative and Inflammatory Diseases of Bones and Joints.* Philadelphia: Lea & Febiger.

KOCH, A. R. (1960). Die Frühentwicklung der Clavicula beim Menschen. *Acta Anat.* **42**, 177–212.

KOSKINEN-MOFFETT, L. & MOFFETT, B. (1984). Influence of prenatal jaw function on human facial development. *Birth Defects; Orig. Article Ser.* **20**, 47–64.

LANSDOWN, A. B. G. (1968). The origin and early development of the clavicle in the quail (*Coturnix c. japonica*). *J. Zool. Lond.* **156**, 307–312.

MURRAY, P. D. F. (1963). Adventitious (secondary) cartilage in the chick embryo and the development of certain bones and articulations in the chick skull. *Aust. J. Zool.* **11**, 368–430.

OGDEN, J. A. (1979). The development and growth of the musculoskeletal system. In *The Scientific Basis of Orthopaedics* (ed. J. A. Albright & R. A. Brand), pp. 41–104. New York: Appleton-Century-Crofts.

OSTROM, J. H. (1976). *Archaeopteryx* and the origin of birds. *Biol. J. Linn. Soc.* **8**, 91–182.

PAI, A. C. (1965). Developmental genetics of a lethal mutation, muscular dysgenesis (*mdg*) in the mouse. 1. Genetic analysis and gross morphology. *Devl Biol.* **11**, 82–92.

RIESENFELD, A. (1966). The effects of experimental bipedalism and upright posture in the rat and their significance for the study of human evolution. *Acta Anat.* **65**, 449–521.

ROMANOFF, H. L. (1960). *The Avian Embryo.* New York: John Wiley & Sons.

ROMER, A. S. (1960). *The Vertebrate Body,* 2nd edn. Philadelphia: W. B. Saunders Co.

RUSSELL, A. P. & JOFFE, D. J. (1985). The early development of the quail (*Coturnix c. japonica*) furcula reconsidered. *J. Zool. Lond.* (A) **206**, 69–81.

THOROGOOD, P. V. (1979). *In vitro* studies on skeletogenic potential of membrane bone periosteal cells. *J. Embryol. exp. Morph.* **54**, 185–207.

TYLER, M. S. & HALL, B. K. (1977). Epithelial influences on skeletogenesis in the mandible of the embryonic chick. *Anat. Rec.* **188**, 229–240.

VINKKA, H. (1982). Secondary cartilages in the facial skeleton of the rat. *Proc. Finnish Dental Soc.* **78** (suppl. 7), 1–137.

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