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The influence of temperature on bone growth in the chick

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INTRODUCTION

It is generally suspected that the physical characters of the osseous circulation are of major importance in the control of bone growth. Normal bones enjoy a blood supply as rich as that of active muscle, and differences in rates of bone formation are correlated with changes in the red cell space and blood flow rate (Brookes, 1971).

Heat is a physical factor second only to oxygen availability in its influence on growth processes in general (Needham, 1964). With regard to bone growth, heat promotes calcification (Fabry, 1954) and bone mineral nucleation (Glimcher, 1960) *in vitro*. Because of a speeding up of intracellular kinetics, a rise in temperature might also be expected to increase matrix production by bone cells *in vivo*. The temperature within bones is inconstant. Fractures feel hot and thermocouple recordings in man (Brodin, 1955; Golenhofen, Hensel & Hildebrandt, 1963) suggest that intra-bone temperature, bone production and blood flow rate are linked together.

Nevertheless, the evidence for a positive heat effect on bone growth in the intact organism is equivocal and almost wholly confined to measurements of tail length in mice reared at high temperatures (Chevillard, Portet & Cadot, 1963; Harrison, Morton & Weiner, 1959), and to the limb length of chick embryos under different conditions of incubation (Romanoff, 1960). Accounts of the efficacy of local applications of heat to lengthen limbs in mammals and children are contradictory (Doyle & Smart, 1963; Granberry & Janes, 1963).

The aim of this investigation was therefore to subject growing bones to temperature change, and to examine its effects on bone production.

MATERIALS AND METHODS

More than 480 fertilized eggs of hybrid white Leghorn hens, produced in one hatchery, were incubated at set temperatures of 35–40 °C, at one degree intervals. The chick embryos were investigated after 10 days incubation.

The incubator, specially designed for this experiment, consisted of an outer commercial biological oven inside which was fitted an airtight Perspex chamber measuring $25 \times 25 \times 25$ cm. The side of the chamber behind the oven door was detachable, and held in place by 12 butterfly nuts and bolts. An air seal was established by the use of a square cut-out rubber washer. The chamber had two built-in trays to hold a dozen eggs, and was also provided with an inlet and an outlet attachment sited below and above the egg trays, respectively. Air was pumped along a polythene tube by means

of a single stroke electric motor, through a water bottle, then through the outer oven casing to the inlet of the inner Perspex chamber. Air was also drawn across the eggs by means of a tube attached to the outlet, and connected to the side arm of a water driven vacuum pump. The temperature inside the egg chamber was monitored by a Compenstat thermostatic probe. The thermostat was also connected to the heating element of the oven. In this way, the temperature of incubation could be steadily maintained with a variation of $< 0{\cdot}05$ °C. The air-flow was held constant at a bubble rate of 15 min observable in the water bottle.

After 10 days incubation, the eggs were removed, and cracked open in a clean glass dish. The chicks were freed of extra-embryonic membranes, and each was washed, weighed and then placed in formal saline. Chicks which were dead, or which showed any gross external abnormality, such as exomphalos, exencephaly, or crossed-bill, were excluded from the experiment. Otherwise, all chicks which were apparently alive and well after 10 days incubation were taken in batches and investigated further, as follows.

The calcium content was measured, by placing each chick in 10 ml of N/10 nitric acid. Pilot experiments showed this always brought about complete decalcification of the skeleton. Samples of the acid extract solution were then estimated for calcium by three different methods, and the total embryonic calcium thereby calculated. The methods employed were oxalate precipitation and titration (Clark & Collip, 1925), colorimetry following the technique of Trinder (1960), and atomic absorption spectrophotometry utilising a Unicam SP 90 apparatus.

Batches of chicks were also prepared in the undecalcified state, stained with alizarin red S, and cleared, as in Dawson's method (1925), for visualization of the bony skeleton. The length of the red stained bone tissue in the humerus, radius, femur, and tibia was then measured on a travelling microscope with a Vernier attachment reading to 0·01 mm. These cleared specimens of the chick skeleton also permitted the total length of the bones to be measured, including their cartilaginous epiphyses.

Some chicks were examined histologically. The tibiae of undecalcified specimens were embedded in paraffin, and small blocks were prepared enabling sections to be cut both transversely and longitudinally on a Jung microtome at 7 μm. These were stained with haematoxylin and eosin, and with Von Kossa's silver stain.

In other specimens, the tibiae were rapidly removed, and fixed in glutaraldehyde and osmium tetroxide solutions. Thin sections were cut with glass knives on a Porter–Blum microtome through the centre of the embryonic shaft. The sections were stained with uranyl acetate and lead citrate (Millonig, 1961), and examined in an EMU 3 (R.C.A.) electron microscope.

RESULTS

Chick wet weight

The mean wet weights of fresh chicks from eggs incubated for 10 days at various temperatures are shown graphically in Fig. 1. The mean at an incubation temperature of 38 °C (2·43 g $\pm$ 0·05) was taken as a reference point for inter-group comparison. The difference between the mean at other temperatures in the graph and at 38 °C was highly significant in all cases ($P < 0{\cdot}001$). The regression coefficient, r, for the line drawn through the means was 0·9911 and the significance of the line was high

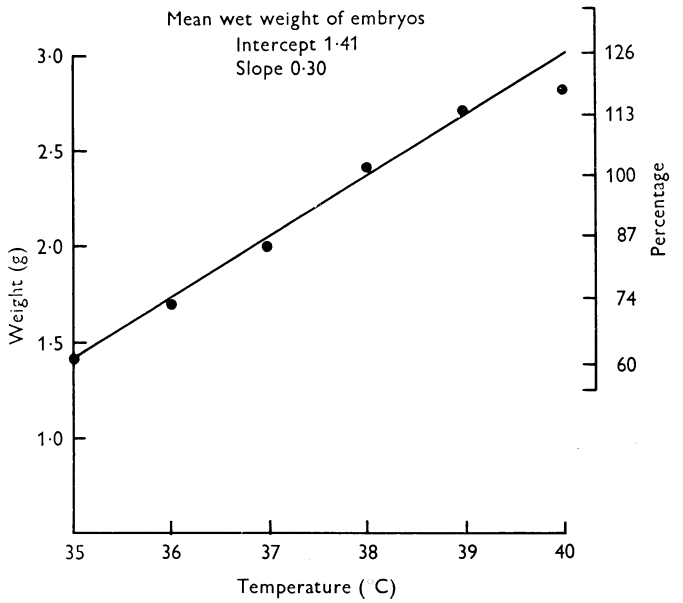

Fig. 1. Graph showing a linear variation with temperature in the weight of chick embryos incubated for 10 days in groups at 35–40 °C. Weight at 38 °C = 100 %. Each point represents the mean of 15 readings.

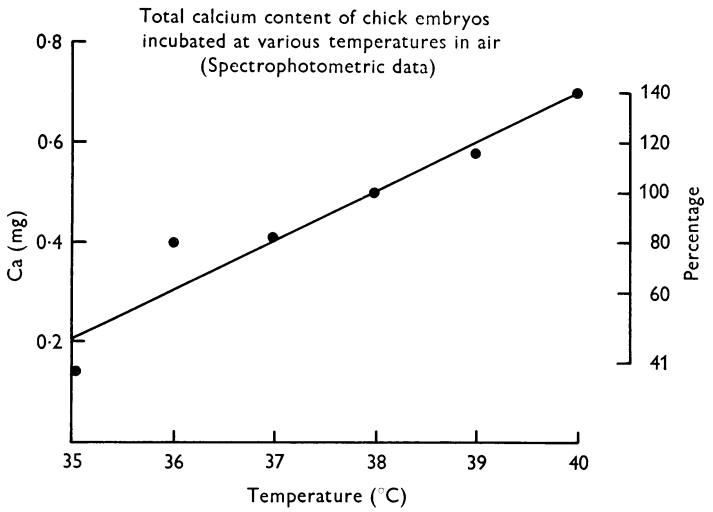

Fig. 2. Regression line drawn through the mean values of total calcium content of chick embryos incubated at 35–40 °C. Data obtained by atomic absorption spectrophotometry.

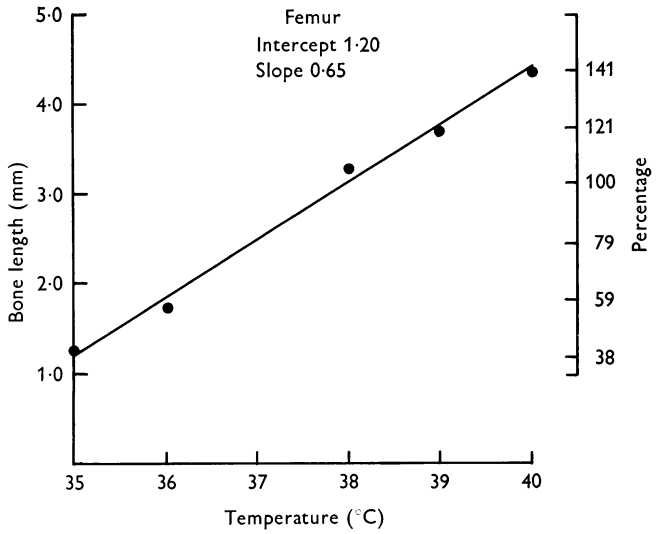

Fig. 3

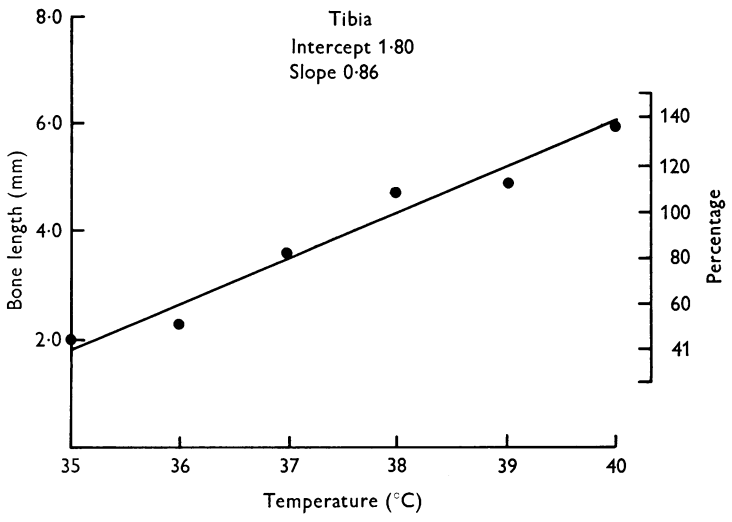

Fig. 4

Figs. 3, 4. Graphs showing variation with temperature in the length of the bony femur (Fig. 3) and tibia (Fig. 4) in 10 day old chick embryos.

($P < 0{\cdot}001$). Fig. 1 therefore indicated that a 1 °C change in temperature was correlated with a 13 % change in chick weight, taking the weight at 38 °C as 100 %.

Calcium estimations

The variation with temperature of chick calcium content measured by spectrophotometry is shown in Fig. 2: a regression line has been drawn through the plotted mean values. Similar graphs were constructed from oxalate precipitation and colori-

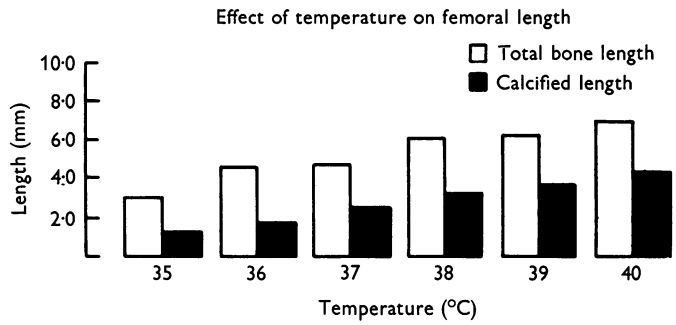

Fig. 5

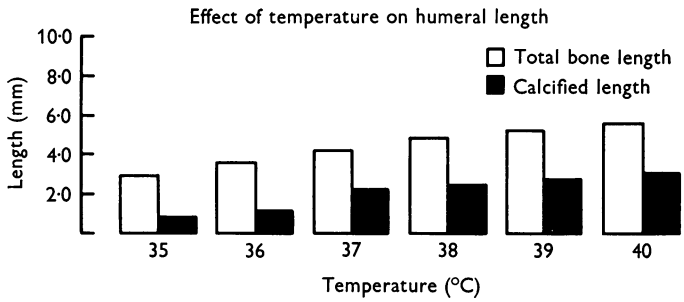

Fig. 6

Figs. 5, 6. Histograms illustrating the linear variation with temperature of the bony length and total length (including cartilage) of the femur (Fig. 5) and humerus (Fig. 6). Data obtained from chick embryos incubated for 10 days.

metric data. The regression coefficients of the lines constructed from these and spectrophotometric data were all highly significant ($P < 0{\cdot}001$). However, the slopes of the lines were radically dissimilar, as were also the mean values for the total calcium of embryos incubated in air at 38 °C (0·2544 mg $\pm$ 0·0068 with oxalate precipitation, 0·5546 mg $\pm$ 0·0187 with colorimetry, and 0·4986 $\pm$ 0·0204 with spectrophotometry). Obviously, the three methods used did not measure calcium with equal accuracy. They, nevertheless, concurred in demonstrating a linear relationship between incubation temperature and chick calcium content. If the most recent and intricate method, atomic absorption spectrophotometry, be taken as the most accurate, then Fig. 2 indicates that, for the 10 day chick, the calcium content rose or fell by 20 % for each °C change in incubation temperature (38 °C = 100 %).

Bone length measurements

The regression lines constructed through the mean values for the length of alizarin red S stained bones, measured with a travelling microscope, are shown in Figs. 3 and 4 in the case of the femur and tibia. Figs. 5 and 6 are histograms to compare the change in total length of bones (including the cartilaginous epiphyses) and in the length of true bone. The regression coefficients for the graphs of true bone lengths as well as those calculated for total length were all highly significant ($P < 0{\cdot}001$). The graphs

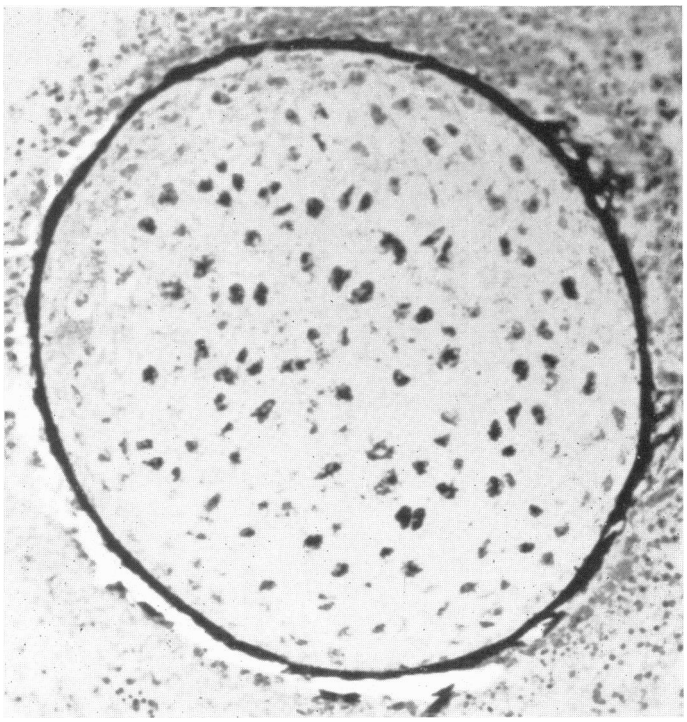

Fig. 7. Transverse section through the undecalcified tibia of a chick embryo incubated for 10 days at 35 °C. Von Kossa's silver stain shows that only the initial periosteal lamella has been formed on the surface of the cartilage primordium. × 125.

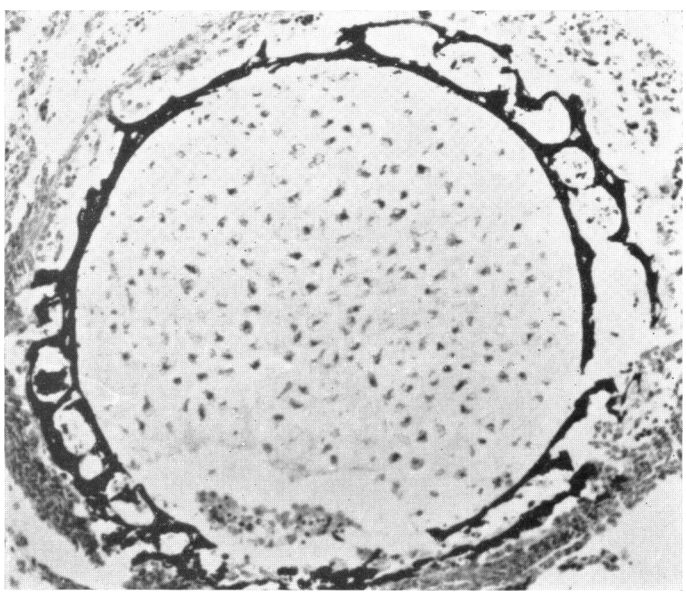

Fig. 8. As in Fig. 7, but with the incubation temperature at 38 °C. The formation of a second ring of bony trabeculae is near to completion. × 66.

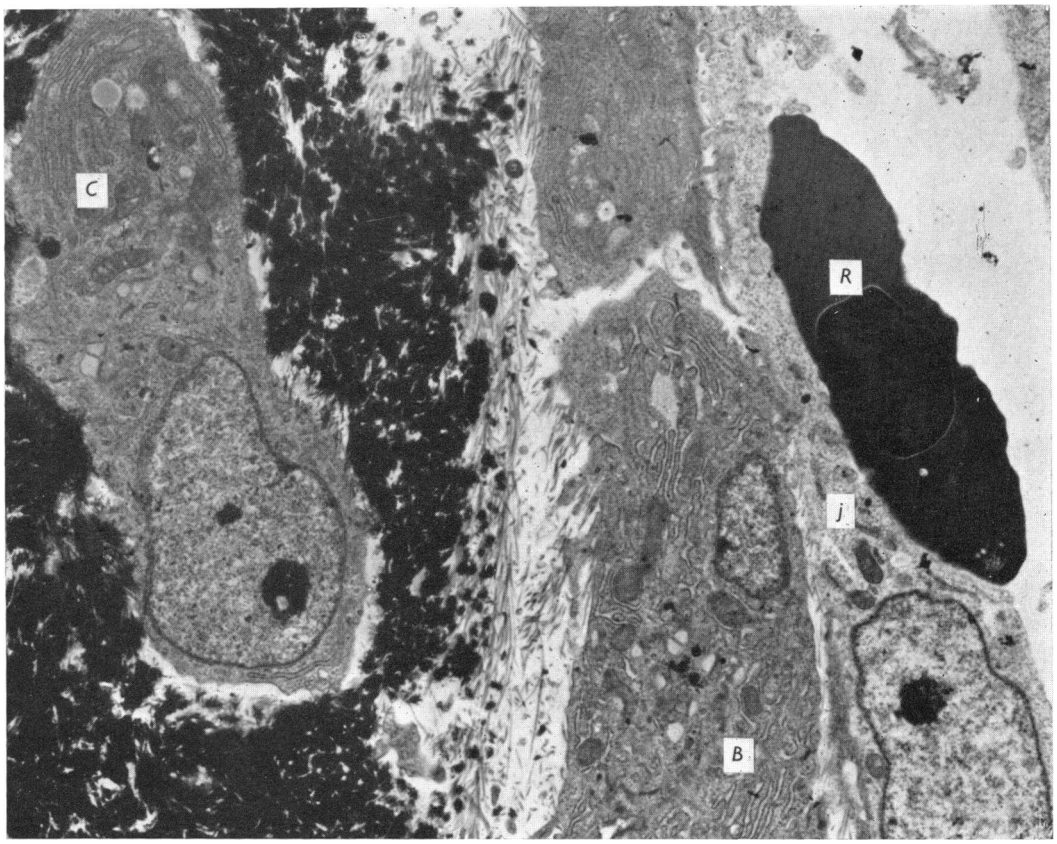

Fig. 9. Electron microscope general view of chick bone incubated at 38 °C, showing a red blood corpuscle (*R*) in a blood vessel. The endothelial cells composing the sinusoid wall are joined by junctional complexes (*j*). Osteoblasts beyond the vessel are shown at *B*. An osteocyte, *C*, is surrounded by dense calcium deposits in a collagenous matrix. (×4000.)

indicate that, for the femur, tibia, humerus and radius, there was a 20 % rise or fall in true bone length for each °C change in incubation temperature (38 °C = 100 %). The change in total bone length (including cartilaginous epiphyses) was 11 % for the humerus and radius, 13 % for the femur, and 14 % for the tibia.

Histological observations

Representative cross sections through the middle of the shaft of chick tibiae incubated at 35 °C and 38 °C are shown in Figs. 7, 8. Bony material is stained jet black by Von Kossa's silver method. It was apparent that only an initial bony ring had formed at the subnormal temperature, whereas a perichondrial collar of bone was considerably advanced at the higher temperature. Tibial cross sections also showed that the constituent trabeculae of the bone present at 10 days incubation were thicker at high temperatures than in low temperature material. Examination of longitudinal sections through chick tibiae also permitted a graded series to be detected in the

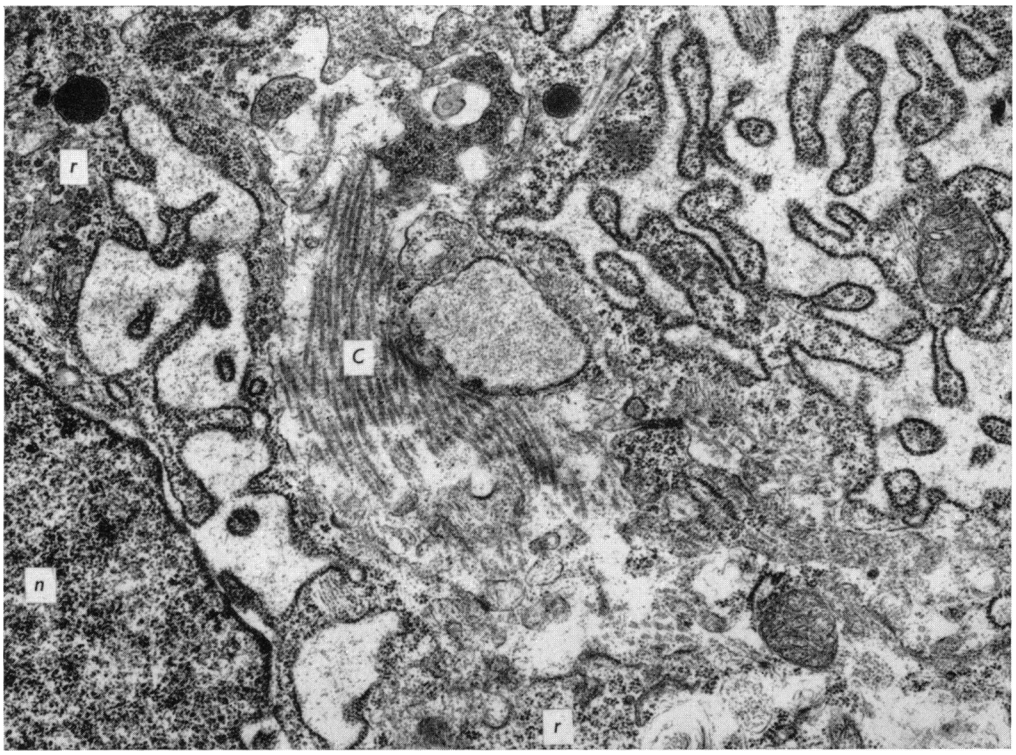

Fig. 10. Electron micrograph of a tangential section through an osteoblast; chick bone, 38 °C. Nucleus, *n*; polysomes, *r*, *r*. The collagen fibres (*C*), are extracellular; the cell membrane around them is indistinct. Mitochondria and cisternae of granular endoplasmic reticulum are conspicuous features of this collagen-producing cell. (× 20250.)

amount of bone formation occurring under the influence of temperature. In particular, histology suggested that the amount of bone present on qualitative examination increased with temperature, supporting the quantitative observations made on calcium content and bone length.

Electron microscope observations

The essential findings are summarized in Figs. 9–13. Bone from chicks incubated at 38 °C (Figs. 9, 10) contained abundant osteoblasts, recognized by their granular endoplasmic reticulum, numerous polysomes, large mitochondria, and an indefinite cell membrane at bone-forming surfaces (Fig. 10). Blood vessels were frequent in E.M. fields. They lay close to calcifying collagen, but never directly adjacent to it, cells of osteoblastic type always intervening (Fig. 9).

At subnormal temperatures (35, 36 °C), the osteoblasts showed a reduction in size of the mitochondria, which were also more electron-dense than normal and somewhat vesiculated in appearance (Fig. 11).

At high temperatures (39, 40 °C) many osteoblast mitochondria were grossly vesiculated (Fig. 12). Osteocyte mitochondria, however, did not show this distortion.

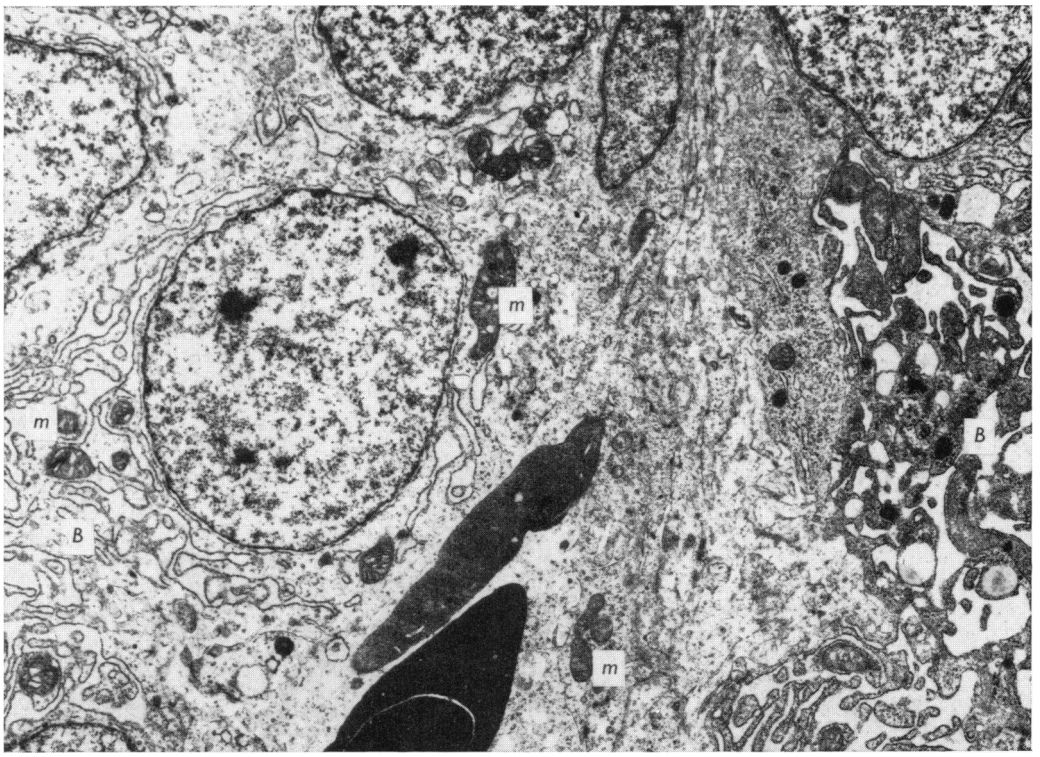

Fig. 11. Electron microscope general view of chick bone incubated at 35 °C. A dense, nucleated red blood corpuscle (characteristic of chick blood) is in the centre, surrounded by osteoblasts (*B*, *B*). The mitochondria (*m*, *m*) are unusually electron-dense. (× 5500.)

At 40 °C, several abnormal cells were also noted (Fig. 13). They differed from bone cells in that they were small, possessed several nucleoli, and contained dense spherical or ovoid inclusions in their cytoplasm. These were identical in appearance to lipofuscin inclusions found in senescent cells elsewhere (Freeman, 1964).

DISCUSSION

The results indicate that bone growth in the chick is accelerated or retarded by a rise or fall in incubation temperature. The temperature/bone growth relationship is linear. For each °C change, longitudinal bone growth is affected by as much as 20 % and the same figure applies to the total calcium content of the embryo.

When considering the effects of environmental temperature change on skeletal growth in the chick, it is to be borne in mind that the avian embryo, like the mammalian embryo, is poikilothermic, and does not possess neurovascular mechanisms for maintaining the internal thermal environment at a high level. It can therefore be assumed with confidence that the temperature of the chick skeleton reflects the external temperature in the incubator.

In the range of temperatures investigated here, it is known that other functions,

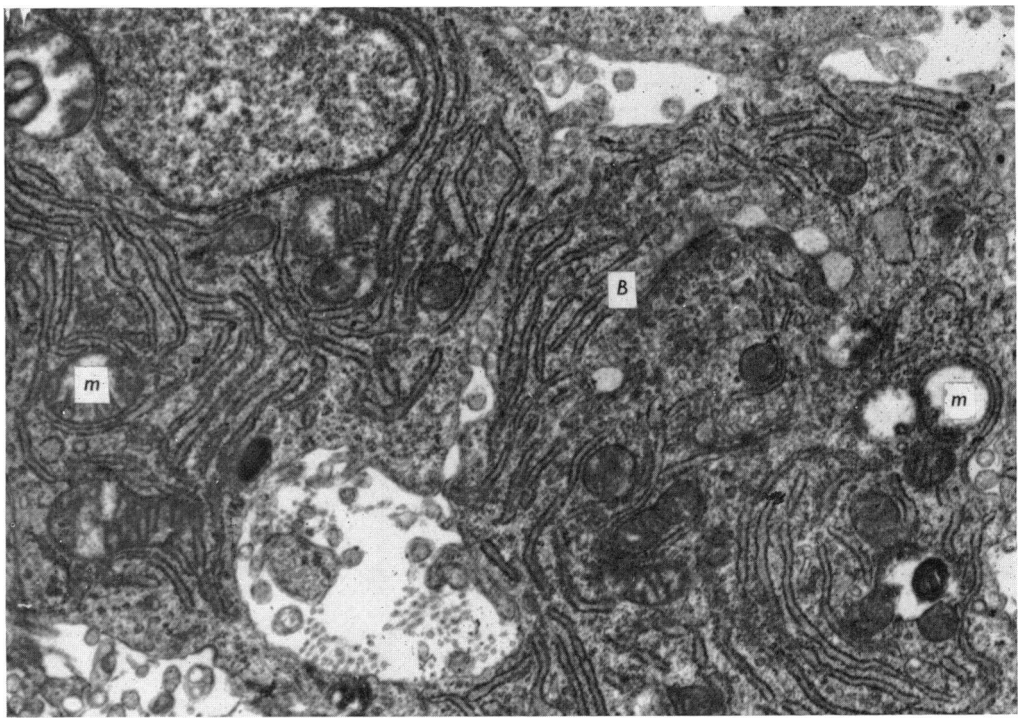

Fig. 12. Electron micrograph of osteoblasts of chick bone incubated at 40 °C. *B* indicates granular endoplasmic reticulum and polysomes. Numerous mitochondria are exhibited; many of them (*m*, *m*) are vesiculated. (×16100.)

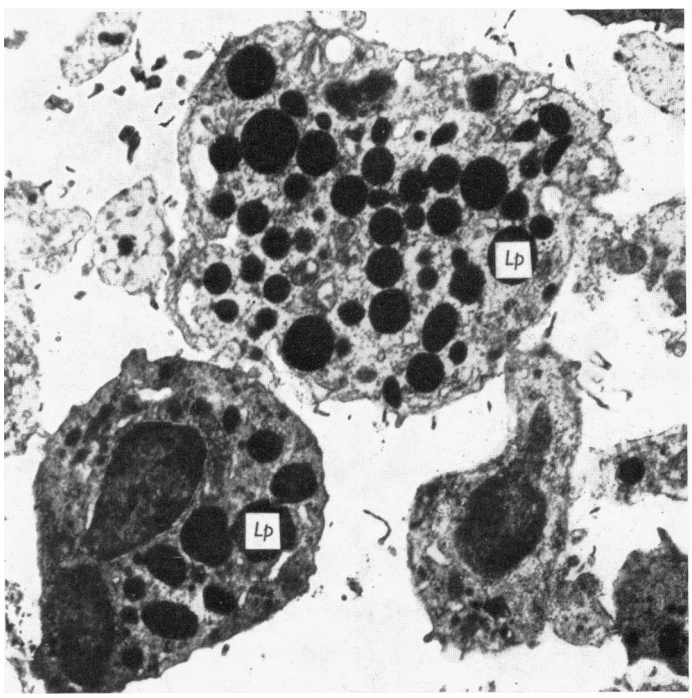

Fig. 13. Abnormal cells found in chick bone incubated at 40 °C, showing electron-dense lipofuscin bodies (*Lp*). (×6400.)

for example, heart rate and oxygen consumption (Phillips, 1941), bear a linear relationship to environmental temperature. Accelerated bone formation with rise in temperature is very much dependent on the integrity of the mitochondria. These are the site of production of ATP molecules, which provide the energy for protein and enzyme syntheses in all cells. They are presumably essential for collagen and protein-polysaccharide synthesis, basic to bone matrix formation by bone cells. The mitochondrial changes observed at high and low temperatures emphasize the importance of these organelles in normal osteogenesis and in the calcification process (Shapiro & Greenspan, 1969). They also suggest that the metabolism of the osteoblasts responds to temperature change directly, and that the effects of bone growth observed in this investigation are the direct result of changed internal temperature acting on the bone cells.

It is most unlikely that the results can be accounted for by increased endocrine activity, because only the primordia of the pituitary and gonads, thyroid and parathyroid are present in the chick at 10 days (Romanoff, 1960), the time at which the experiments were terminated. Furthermore, according to Thomas (1960), the avian glands only enter into activity shortly before hatching (22 days). The results can in part be explained by earlier or later appearance of ossification at high or low temperatures, so that the amounts of bone observed at 10 days at different temperatures, correspond to different developmental stages in the chick as a whole. This undoubtedly accounts to a considerable extent for the increasing quantities of bone observed with increasing temperature. The results show that the chick whole weight and the total length of the humerus, radius, femur and tibia all vary with temperature about 12% per °C. Nevertheless, this measure of general and skeletal development in the observed temperature range contrasts with the 20% per °C variation in calcium content and true bone lengths. The linear increase in bone growth with rise in temperature observed in this investigation results therefore not only from an acceleration of skeletal development but also of bone growth. At a given developmental stage, there is more bone present at high temperatures.

From a cellular point of view, bone formation in a chick embryo is much the same as bone formation in mammals. Hence it is useful to compare the results obtained here in avian material with those found in human therapeutics and in mammalian experiments designed to lengthen bones by the local application of heat. Bertrand & Trillat (1948) and Doyle & Smart (1963) obtained growth-promoting effects, in children and rats respectively, by heating the knee joint with short wave diathermy. Richards & Stofer (1959) prepared an electrical hot wire sling around the femora of dogs and rats, and reported increased longitudinal bone growth after the use of this drastic method of bone heating. Other investigators, however, have been unsuccessful in the use of heat as a growth-promoting agent (Forest *et al.* 1953; Granberry & Janes, 1963). The experiments of all these workers differ fundamentally from what happens in the living chick. Here a rise in bone temperature is accompanied by an increased blood supply, but when heat is applied locally to a joint, an increase in the blood supply would appear to be fortuitous. Further investigation is required in order to specify in therapeutics the precise mode of application and degree of heat that will, with certainty, bring about the dilatation of bone arterioles and thus increase the blood flow rate. Bone cells, whatever the stimulus which excites them into

increased activity, will not produce more bone if an increased supply of bone building materials is not at hand, whether in an organ culture medium or in circulating blood.

During the course of fracture repair Brookes, Richards & Singh (1970) have measured a massive increase in blood flow rate to soft bony callus while it is forming. The method of flow-rate measurement used by these workers measures the rate of input of arterial blood, so that a rise in temperature of the healing fracture is highly probable. The rise, even if small, would seem, on the evidence from avian material, to be a factor of some importance in promoting the growth of new bone trabeculae in callus formation during fracture repair in man. The same might also apply to the accelerated bone formation which occurs in cases of arteriovenous fistula and in Paget's disease, where there is believed to be an increased flow of arterial blood to the affected part.

SUMMARY

1. A convenient incubator was designed and constructed for accurate control of the environmental temperature of incubating chick eggs. After 10 days incubation in the range 35–40 °C, batches of eggs were opened, and the embryos weighed and investigated with respect to skeletal growth.

2. The results indicate that the total skeletal calcium and the true bone length of several long bones vary linearly with the temperature of the chick embryo by 20% per degree centigrade. Chick development and total bone length (including the cartilaginous epiphyses) vary only 12%. It is argued that heat not only promotes skeletal development, but accelerates bone formation to a major extent.

3. It is suggested that a rise in temperature brought on by a raised arterial input may be a relevant factor in accelerated bone production in mammals, for example, in fracture repair and in human leg lengthening by means of an arteriovenous fistula.

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