The Growth of Cartilaginous Embryonic Chick Bones After Freezing

I. *Introduction.*—Although considerable progress has been made in the development of techniques for freezing cells and tissues, attempts to preserve adult human cartilage at temperatures below 0°C have failed[1]. Since cartilage undergoes considerable modification in its structure and metabolism during maturation the effect of freezing on embryonic cartilage is of interest, and in this paper the results of preliminary work on the freezing of cartilaginous tibiae from 6–7 day old chick embryos is described. These cartilaginous rudiments grow and differentiate in tissue culture[2], and therefore their response *in vitro* provides a very convenient test of viability after thawing.

II. *Material and Methods.*—The tibiae from 6 and 7 day old chick embryos were used in this work. The bones contained in small test tubes were cooled to −79°C in about 40 min using the freezing technique described by SMITH[3]. After the requisite period at −79° Celsius had elapsed the bones were rapidly thawed by immersion of the tubes in a water bath at 37°C. The bones were then removed, rinsed in Tyrode solution and transferred to the surface of the clotted plasma in the culture dishes.

The explants were cultivated at 37°C using the watch glass method for solid media[4], some bones being kept for 3 days and others for 6 days. If the test lasted more than 3 days the medium was renewed. The medium consisted of 0·01 ml of 6·5% glucose solution, 0·375 ml fowl plasma and 0·125 ml of an extract in Tyrode of a minced chick embryo. The extract was prepared from a 13 day embryo in the first experiment and from a 9 day embryo in the second.

Two treatments were used in each experiment and these were allotted at random to one or other of the two tibiae from each embryo. Drawings were made with a camera lucida of the bones initially and again on the first and sixth days of cultivation. From these the length and maximum width of each epiphysis were determined. The average epiphyseal width of each bone is defined as the mean of the two maximum epiphyseal widths. The statistical analyses follow standard procedures and have been made after transformation of the data to the logarithmic scale.

III. *Results.*—(1) The tibiae from five chick embryos (7 days' incubation) were used in this experiment. The two treatments compared were (*a*) storage at −79°C for 1·5 h after freezing in 15% glycerol saline, and subsequent culture, and (*b*) culture without freezing (control treatment).

[1] R. C. CURRAN and T. GIBSON, Proc. roy. Soc. [B] *144*, 572 (1956).

[2] H. B. FELL, *Skeletal development in tissue culture* in *The biochemistry and physiology of bone* (Ed. G. H. Bourne, Academic Press, New York 1956).

[3] A. U. SMITH, Exp. Cell Res. *3*, 574 (1952).

[4] H. B. FELL and R. ROBISON, Biochem. J. *23*, 767 (1929).

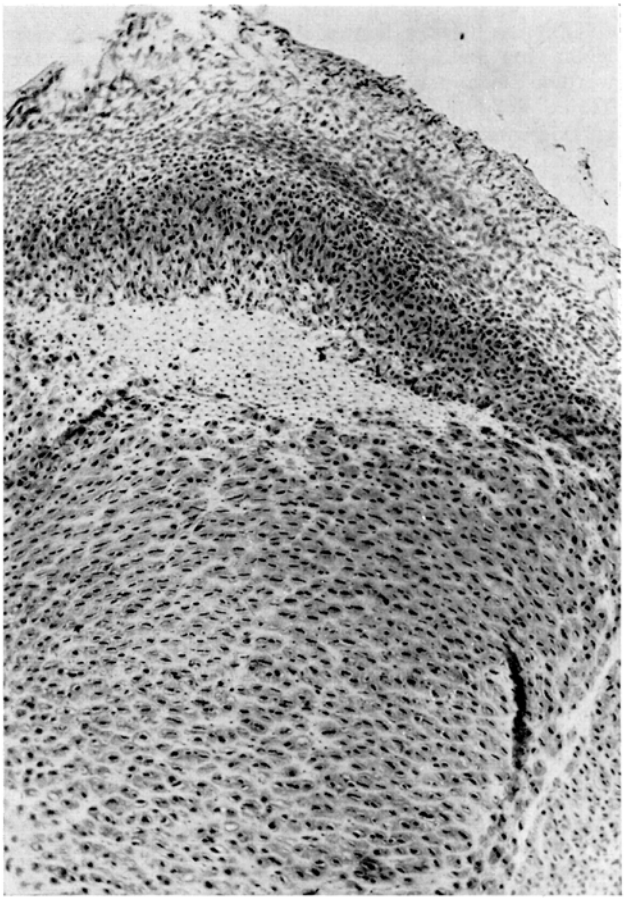

Fig. 1.—Epiphysis of a tibia cultivated for 3 days after freezing. × 126.

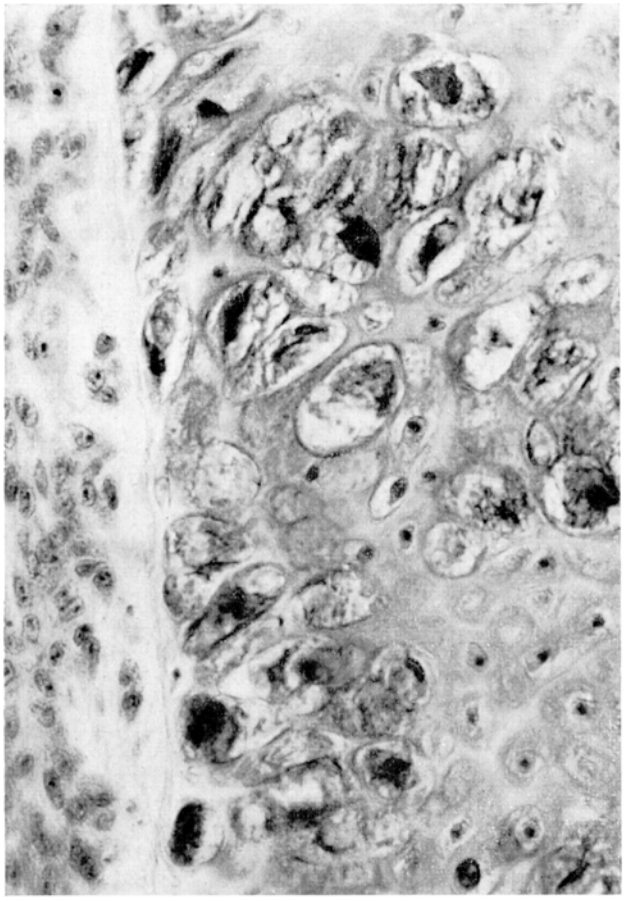

Fig. 2.—Diaphysis of a tibia cultivated for 3 days after freezing. × 500.

The effect of freezing on the length and average epiphyseal width of 7 day old embryonic chick tibiae after 3 days' cultivation.

Treatment	Mean log length		Difference (d)
	Initial	Final	
Unfrozen . .	0·616	0·800	0·184
Frozen . . .	0·622	0·676	0·054

Standard error of d: 0·015, 8 D.F.

Treatment	Mean log epiphyseal width		Difference (d)
	Initial	Final	
Unfrozen . .	0·124	0·200	0·076
Frozen . . .	0·122	0·206	0·084

Standard error of d: 0·012, 8 D.F.

The measurements obtained from the camera lucida drawings made after 3 days' cultivation are summarized in the Table. The results demonstrate that although the frozen bones elongated to a significant degree ($t_{(8)} = 3{\cdot}60$, $0{\cdot}01 > P > 0{\cdot}001$), the extent of the elongation was much less than that observed on the unfrozen controls. The Table also shows that considerable and equal growth of the epiphyses occurred in both the frozen and unfrozen bones.

Histological examination of the explants showed that the majority of the rounded epiphyseal and flattened cells survived the freezing, although in most specimens some cells in the centre of the epiphysis were dead (Fig. 1). In contrast, however, a large number of the cells in the diaphysis were dead. A few were alive and hypertrophied, but they appeared very distorted (Fig. 2). The periosteum and the zone of periosteal ossification was normal. In some specimens, cultivated for 6 days after freezing, mitotic figures were found in the outgrowth of fibroblasts, the periosteum and among the chondrocytes of the epiphysis.

(2) The tibiae from six chick embryos (6 days' incubation) were employed. The two treatments were storage for 1 h at −79°C and subsequent culture after (*a*) freezing in normal saline, and (*b*) freezing in 15% glycerol saline.

After 3 days' cultivation all the bones frozen in normal saline were dead. All the bones frozen in glycerol saline, however, survived the freezing. The degree of elongation varied from specimen to specimen. Two of the bones doubled their length and remained normal in shape, while the others elongated to a lesser degree accompanied by some distortion. In all specimens the epiphyseal and flattened cells were normal in appearance and showed mitotic activity. No necrosis was seen in these regions. Only a few cells were dead in the diaphysis, the majority being hypertrophic. In some regions of the diaphysis, however, the matrix was abnormal in structure, although it stained metachromatically with toluidine blue.

IV. *Discussion.*—The results show that it is possible to freeze, and subsequently revive, all parts of the cartilaginous tibiae from 6 day old chick embryos, provided glycerol is present in the freezing medium. In contrast, only the epiphysis of the tibiae from 7 day old embryos survive to any extent. Thus, during a period of 24 h normal growth and differentiation the majority of the cells in the diaphysis become sensitive to the effects of freezing and are not protected by glycerol. At this stage of embryonic development the tibia undergoes rapid differentiation, the most spectacular change being the hypertrophy of the diaphyseal cells.

The causes of cellular damage on freezing, and the mechanism of the protective action of glycerol which is found with almost all tissues studied, has been analysed from a general point of view by LOVELOCK[5]. It is conceivable that the failure of glycerol to protect the diaphyseal cells is due to increased susceptibility to raised electrolyte concentrations or to the high glycerol concentrations. Changes in the permeability of the cell membranes may also be involved. Alternatively, the formation of matrix may retard the penetration of glycerol.

The results of freezing tibiae from 7 day old embryos is also of interest from a morphogenetic point of view, for the effect is to destroy differentially the zone of hypertrophic diaphyseal cells. In this zone, unlike the zones of epiphyseal and flattened cells, mitoses are rarely seen[6]. Thus, the fact that elongation of the bone is suppressed when the hypertrophic zone is destroyed demonstrates experimentally that the process of hypertrophy plays an important role in determining the shape of the developing cartilaginous rudiment.

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Zusammenfassung

Tibien von sechs Tage alten Hühnerembryonen überleben eine Abkühlung auf −79°C. Im Gegensatz dazu werden die hypertrophischen Zellen der Diaphyse von Tibien siebentägiger Embryonen durch Gefrieren weitgehend zerstört, während der Rest der Rudimente überlebt.

[5] J. E. LOVELOCK, *Biophysical aspects of the freezing of living cells* in *Preservation and transplantation of normal tissues* (Ed. G. E. W. Wolstenholme & M. P. Cameron; Churchill, London 1954).

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Uracil Metabolism in *Neurospora crassa*

That a reductive pathway for pyrimidine catabolism exists was first suggest by FINK *et al.*[1]. Later, by tracer experiments *in vitro* and *in vivo*[2], by chromatography[3], and by enzyme studies[4], it was established beyond doubt that thymine or uracil may be degraded to β-amino acids via the corresponding dihydropyrimidines and β-ureido acids in animal tissues.

[1] K. FINK, R. B. HENDERSON, and R. M. FINK, J. biol. Chem. *197*, 441 (1952).

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[3] R. M. FINK, K. FINK, and R. B. HENDERSON, J. biol. Chem. *201*, 349 (1953). – R. M. FINK, C. MCGAUGHEY, R. E. CLINE, and K. FINK, J. biol. Chem. *218*, 1 (1956). – K. FINK, J. biol. Chem. *218*, 9 (1956).

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