CLXXXII. THE POSSIBLE SIGNIFICANCE OF HEXOSEPHOSPHORIC ESTERS IN OSSIFICATION.

PART V. THE ENZYME IN THE EARLY STAGES OF BONE DEVELOPMENT.

BY MARJORIE MARTLAND AND ROBERT ROBISON.

From the Biochemical Department, Lister Institute, London.

(Received November 1st, 1924.)

PREVIOUS experiments [Robison, 1923], which demonstrated the presence of a phosphoric esterase in the bones of young animals, failed to detect this enzyme in unossified cartilage. The present work was undertaken to confirm this observation, and to determine whether any correlation could be traced between the appearance of the enzyme and the beginning of the ossification process in the cartilage.

Our first experiments were carried out on the cartilages of a full-term infant who died 43 hours after birth. Extracts were made (*a*) from the costochondral junctions, including half-an-inch of bone and of cartilage on either side of the line of ossification, (*b*) from the costal cartilages at their sternal ends, and (*c*) from the patellae. In (*b*) and (*c*) no sign of commencing ossification could be made out.

The parts to be examined were dissected free from muscle or ligament, cut into thin slices, and ground up with twenty times their weight of distilled water saturated with chloroform, in which they were allowed to remain for 48 hours at room temperature. These 5 % extracts were then tested, without filtration, for the presence of the enzyme, using sodium glycerophosphate as the substrate. A number of tubes was prepared, each containing

10.0 cc. *M*/1000 sodium glycerophosphate solution.

1.0 „ 5 % tissue extract.

0.1 „ phenolphthalein, alkali, etc., to adjust the p_H to 8.4.

0.1 „ chloroform.

These were kept in a thermostat at 38° and at the stated intervals a tube was removed and the inorganic phosphate estimated by the Briggs method after precipitation of the proteins with trichloroacetic acid. The results as shown in the tables give the amount of hydrolysis effected by 1 cc. of the 5 % extracts. Control tubes containing tissue extract + water, and substrate + water, were simultaneously examined, but as these showed no increases in inorganic phosphate the figures have been omitted from the tables.

Table I. *Cartilages of full-term infant.*

Hydrolysis of <i>M</i> /1000 sodium glycerophosphate (10 cc. = 0.31 mg. P.) effected by 1 cc. 5 % extract at 38°, p_H 8.4. mg. P.		
Source of 5 % extract.	3 hours	18 hours
Costochondral junction	0.066	0.182
Costal cartilage, unossified	0.006	0.006 ¹
Patella, unossified	0.009	0.009 ¹
Bones of young rats (control)	0.106	0.264

We next investigated the bones of a human foetus of 5½–6 months' development. The femora, tibiae and humeri were dissected out and divided into three portions, (a) completely ossified shaft, (b) the epiphyseal cartilages in which no ossification was apparent, and (c) the junction between (a) and (b) including the line of ossification. In the case of the metatarsals and of the pelvic bones the separate centres of ossification were dissected as completely as possible from the unossified cartilage. Extracts were made of these various parts and tested in the manner detailed above, except that in this case the concentration of the sodium glycerophosphate was *M*/100. An extract of the parietal as an example of a membrane bone was also prepared and tested.

Table II. *Skeleton of a 5½ months foetus.*

Hydrolysis of <i>M</i> /100 sodium glycerophosphate (10 cc. = 3.1 mg. P.) effected by 1 cc. 5 % extract at 38°, p_H 8.4. mg. P. in		
Source of 5 % extract.		18 hours
Femur	Shaft (ossified) ...	1.85
	Junction ...	2.20
	Epiphysis (unossified)	0.010
Tibia	Shaft (ossified) ...	1.46
	Junction ...	1.74
	Epiphysis (unossified)	0.013
Humerus	Shaft (ossified) } ...	2.08
	+ Junction }	
	Epiphysis (unossified)	0.006
Metatarsals	Ossifying centres ...	0.303
	Cartilage (unossified)?	0.095
Ileum }	Ossifying centres ...	1.76
Ischium }		
Pubis }	Cartilage (unossified)	0.006
Parietal	Ossified ...	1.28

Both these experiments show that while the ossified portions of the young bones contained the phosphoric esterase in a high degree, the enzyme was not present in significant amount in the epiphyses of the long bones, in which the ossification centres had not yet appeared, nor in the costal cartilages, nor in the unossified cartilage of the pelvic bones. The patella which alone was entirely cartilaginous also contained none of the phosphoric esterase.

¹ The identity of these figures with those for 3 hours suggests that this small increase in the inorganic phosphate was not due to enzymic hydrolysis of the ester.

The metatarsal cartilages in Exp. 1 appeared at first to give an exceptional result, but a close examination of the parts¹ from which the extract had been made showed that some of the minute adjacent bones (phalanges), which contained ossifying centres, had been included and would account for the enzymic activity of the extract. It is interesting also to note the presence of the enzyme in membrane bones (parietals) as well as in those developed from cartilage.

The evidence of these experiments is sufficient proof that in partially ossified bones the enzyme is present only in those parts where active ossification is proceeding. We wished to carry the argument a stage further and to show that in one and the same bone, the first appearance of the enzyme coincides with the appearance of the ossification centre. We were here met by the difficulty of obtaining a human foetus at a sufficiently early stage of development for our purpose. The centres of ossification appear, for the most part throughout the human skeleton, during the second and third months of foetal life, and bone formation proceeds with such rapidity that within the course of a few weeks the greater part of the shafts of the long bones becomes calcified. The earliest foetus we succeeded in obtaining, of about 2½–3 months' development already showed extensive ossification throughout the long bones and ribs. We therefore sought for some bone which might be obtained in an entirely unossified state. This requirement is fulfilled by the patella, which remains entirely cartilaginous until three years or more after birth, when a single centre of ossification appears, from which bone formation spreads slowly, becoming complete at about puberty. We have compared the hydrolytic activity of cartilaginous and ossifying patellae obtained post mortem from children of varying ages. The following notes give details of the material dealt with and the presence or absence of phosphoric esterase, the experimental figures being shown in Table III.

A. 5 % extract of patellae from two still-born children; both entirely unossified. No phosphoric esterase was present.

B. 5 % extract of patella from a 7 months old child; entirely unossified. No phosphoric esterase was present.

C. 5 % extract of patella from a boy aged 5 years. The patella was bisected antero-posteriorly in the vertical plane, and the half from which the extract was made showed no ossification. No phosphoric esterase was present. The other half, kept for histological examination, was subsequently found to contain a well-developed centre of ossification, eccentric in position. As this ossifying half had been soaked in formalin for two days, the presence of an enzyme was not to be expected, nor was any found in the extract made therefrom.

D. 5 % extract of patella from a girl aged 6 years. The patella was bisected

¹ Fortunately in this case, owing to the minute amount of the tissues, these had not been macerated but merely allowed to soak in the water. It was thus possible to detect the phalanges with the aid of a lens.

antero-posteriorly in the vertical plane. The cut surface, which measured 32×16 mm. showed a well-developed centre of ossification, central in position and oval in shape, measuring 10×5 mm. Early ossification was also seen just deep to the periosteum, extending radially towards the centre of the bone. The extract was prepared from one half of the patella and although this included a large proportion of cartilage the extract showed a very considerable enzymic activity.

Table III.

Hydrolysis of $M/100$ sodium glycerophosphate
effected by 1 cc. 5 % extract at 38° , pH 8.4.
mg. P.

Source of 5 % extract.	mg. P.	
	3 hours	18 hours
A. Unossified	0.000	0.000
B. Unossified	0.000	0.000
C. Unossified half	—	0.002
D. Partly ossified	0.035	0.220

We have thus clear proof that prior to the appearance of the centre of ossification the patella does not contain the phosphoric esterase but that this enzyme is present soon after ossification has commenced.

SUMMARY.

An investigation of cartilages, and of bones in early stages of development, from human embryos and young infants has shown that the enzyme which hydrolyses monophosphoric esters is not present in any cartilage prior to the appearance of a centre of ossification but is found wherever active ossification is taking place.

It therefore seems legitimate to conclude that the two phenomena are bound up together and that the production of the enzyme is a part of those cellular activities which result in the formation of bone.

We are greatly indebted to Dr R. T. Brain of the London Hospital and to Dr E. H. Lepper of the Elizabeth Garrett Anderson Hospital for their help in supplying the material for these experiments.

REFERENCE.

Robison (1923). *Biochem. J.* **17**, 286.