DISCUSSION

The comparatively laborious procedure of purification of arginase, as described by Bach & Killip (1958, 1961), has been considerably simplified, so that the enzyme can now be extracted from acetone-dried ox-liver powder and purified to the precrystallization stage in less than 2 days of laboratory work. By elaborating the mode of the ammonium sulphate fractionation, the method has become easily reproducible and may lend itself to teaching experiments. From the final stage of the purification procedure, the enzyme can be crystallized in one further step, by using the procedure reported by Bach & Killip (1958).

SUMMARY

1. A comparatively short method for the preparation of arginase from ox liver is described which permits the purification of the enzyme from acetone-dried liver powder to the precrystallization stage in 2 days of laboratory work.

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The Distribution of Organically Bound Sulphate in Bone and Cartilage during Calcification

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Wherever calcification takes place, sulphated mucopolysaccharides seem to be present. They are found in sites of osteogenesis (Kent, Jowsey, Steddon, Oliver & Vaughan, 1956), are a principal constituent of calcifying cartilage (Dziewiatkowski, Di Ferrante, Bronner & Okinaka, 1957), and occur during dentine formation (Léblond, Bélanger & Greulich, 1955). As a result of this, sulphated mucopolysaccharides have for many years been linked with the processes of hard-tissue formation.

Despite the considerable coincidence between the occurrence of these sulphate esters and the deposition of mineral salts, their role in the process of calcification is still not sufficiently understood. There is disagreement whether these substances assist calcification, or prevent it. Some workers have postulated that their presence is associated with the initiation of calcification (Rubin & Howard, 1950; Sobel, Burger & Nobel, 1960), whereas others (Logan, 1935; Sylvén, 1947; Glimcher, 1959) suggested that their removal promotes mineralization of the tissue.

The changes that occur in the concentration of organically bound sulphate in a tissue during its mineralization have not been clearly established, and the present work was undertaken to obtain information about the concentrations of these substances in different regions of calcifying epiphyseal cartilage of ox foetus.

MATERIALS AND METHODS

Epiphyseal cartilage from both ends of the femora of foetal ox was selected for examination. The foetuses chosen were approx. 45 cm. in length, nose to anus, taken from mixed breeds of cattle at the Leeds City Abattoir. The cartilaginous epiphyses were removed from both ends of the femora, and either dissected and analysed immediately or deep-frozen until use. The epiphyses contained no secondary centres of ossification and their large size permitted a number of analyses to be carried out in a continuous series from the non-calcifying cartilage of the hyaline region to the primary bone spicules of the metaphysis.

Distribution of samples. After the epiphyseal cartilage was removed from the femora, the outer surface of articular cartilage was cut away and the remaining material sliced sagittally into sections 0.5 mm. thick. From these sections, narrow strips of cartilage parallel to the growth plate were cut under the dissecting microscope and pooled into samples representing six regions of cartilage as shown in Fig. 1. The first sample, *A*, represented the uncalcified hyaline region of the epiphyseal head. Hyaline cartilage was usually separated from the growth plate by a narrow band of optically dense tissue, *B*, rather similar in appearance to hyaline cartilage but less basophilic and perhaps more cellular. This area, which will be called the 'dense' region, constituted the second sample. The third, fourth and fifth samples, *C*, *D* and *E* respectively, were adjacent areas of the epiphyseal plate, the third being next to the dense area, and the fifth lying against the translucent region of hypertrophied and degenerate chondrocytes. The sixth area, *F*,

was the translucent region itself, constituting a narrow strip next to the calcification front, where cartilage abutted on to the metaphyseal spicules of intramembranous bone. This sample unavoidably contained some remnants of bone spicules attached to it, of which as much as possible was removed before analysis.

In addition to the samples of cartilage, four specimens of bone were taken from some of the femora. The first two, *G* and *H* respectively, were immediately adjacent to the epiphyseal plate and presumably contained small amounts of entrapped remnants of calcified cartilage. The third sample, *I*, was a specimen of cancellous bone taken from the medullary cavity as far from the epiphysis as possible. The fourth sample, *J*, consisted of shavings of periosteal bone from the mid-shaft of the diaphysis.

Decalcification of samples. The dissected bone and cartilage were cleaned by several washings in 0.9% sodium chloride and decalcified overnight at room temperature with 1% nitric acid. After being washed, they were dried at room temperature *in vacuo* over phosphorus pentoxide.

Sulphate determination. The dried specimens of decalcified bone and cartilage were weighed and oxidized by nitric acid digestion in the presence of excess of cation (K^+ or Na^+). After evaporation to dryness, the residue was dissolved in 4% (w/v) trichloroacetic acid, so that a 4 ml. sample of the solution contained 1.5 mg. of cartilage, i.e. approx. 90 $\mu g.$ of SO_4^{2-} ion. Sulphate was determined by Dodgson's (1961) method. The recovery of known amounts of inorganic SO_4^{2-} ion, passed through the entire procedure, was within 3%. The S.D. of quintuple determinations of SO_4^{2-} ion on the same sample of cartilage was about $\pm 2.5\%$. The results obtained from bone were less satisfactory. A series of seven determinations carried out upon a single ground sample of compact human femoral bone gave results within 5% of the mean, but variations of up to 20% occurred between individual samples of bone which were apparently similar. This was presumably partly due to greater biological variation but also to occasional failure to remove completely the Ca^{2+} and PO_4^{3-} ions from the matrix.

RESULTS

Average values for the percentage of organically bound sulphate, expressed on a dry-weight basis, are presented in Fig. 1. This relates the analysis to the region from which the samples were taken.

The concentration of organically bound sulphate is approximately the same in the calcifying epiphyseal plate as in the non-calcifying hyaline region. The dense region, which is the area least stained by toluidine blue, has an accordingly low sulphate content. This would be expected since chondroitin sulphate is markedly basophilic. Within the epiphyseal plate itself there is, if anything, a slight increase in the concentration of sulphate towards the calcification front, followed by a decrease in the hypertrophic and degenerate region. This decrease is most likely due to the difficulties in sampling rather than to a change in the sulphate content of cartilage. It is not possible to dissect the narrow strip of degenerate cartilage

Fig. 1. Organically bound sulphate in dry decalcified epiphyseal cartilage, metaphyseal and diaphyseal bone of the femur of calf foetus. Samples were taken from: A, hyaline cartilage of epiphysis; B, 'dense' region of cartilage; C, D and E, adjacent areas of epiphyseal plate; F, hypertrophic and degenerate cartilage; G, metaphyseal bone; H, metaphyseal bone; I, cancellous bone from diaphysis; J, periosteal compact bone from diaphysis. Values are expressed as g./100 g. of dry decalcified material. The section shows epiphyseal cartilage stained with toluidine blue.

cells without including in the sample some of the attached bone fringe, and the degree of contamination of cartilage by bone matrix cannot be controlled by any chemical determination. In the next region there is an abrupt decrease in the concentration of sulphate coincident with the production of metaphyseal bone by invading osteoblasts. The decrease in the concentration of sulphate continues in the samples taken from more mature bone.

DISCUSSION

The sulphate of decalcified cartilage is present in the form of acid mucopolysaccharides, principally chondroitin sulphate.

The percentage of sulphate is fairly constant throughout the epiphyseal cartilage, apart from the relatively low concentration found in the optically dense region, which corresponds to the lightly stained region of Fig. 1 and is situated between the hyaline cartilage and the epiphyseal plate.

Several workers have suggested that the deposition of bone salt is associated with a removal of

organic sulphate from cartilage. The analyses presented here do not support this view. Even the region of cartilage cells that is closest to the fringe of metaphyseal bone trabeculae possesses a sulphate content almost as high as that of hyaline cartilage. It seems very likely that the values for the sulphate concentration of this degenerate region would have been higher still if the sample had consisted entirely of cartilage uncontaminated by bone matrix. Unfortunately, a complete separation of cartilage from osteoid was impossible. The region of hypertrophic and degenerate chondrocytes in the calf epiphysis inevitably included a visible fringe of metaphyseal bone spicules and, therefore, an unknown percentage of calcified osteoid with a comparatively low content of organically bound sulphate. The decrease in the sulphate content of this region of hypertrophic and degenerate cells could be accounted for by the inclusion of as little as 10 % of intramembranous bone matrix. It seems reasonable to assume that the lowering of the sulphate concentration is due to the presence in the sample of small amounts of decalcified bone, rather than to a loss of sulphate from cartilage.

After the cartilage matrix calcifies, intramembranous bone is laid down on the endochondral spicules. The isolated remnants of cartilage are gradually resorbed and replaced by newly formed bone. The samples of bone matrix contained less sulphate than cartilage, and the precipitous fall in the concentration of organically bound sulphate appears to result from the replacement of endochondral elements by intramembranous bone. As further remnants of cartilage are removed, the sulphate content gradually falls to the level found in the samples of mature cortical and cancellous bone.

Although the area of the earliest mineral salt deposits is difficult to define, the present findings do not indicate that this coincides with a rapid disappearance of sulphated mucopolysaccharides. Calcification is well advanced in the metaphyseal aspect of the translucent area of hypertrophic and degenerate cells, where a fringe of bone is clearly

visible, and where the first slight fall in sulphate content is found. Electron micrographs of kitten epiphyseal cartilage (Scott & Pease, 1956) indicate that the first mineral is laid down in the zone of cartilage before the degeneration of the cells, which is some distance away from the metaphyseal spicules. This tissue area contains a comparatively high concentration of organically bound sulphate.

SUMMARY

1. The distribution of sulphate fixed in the organic matrix of calcifying cartilage and bone from the femur of the foetal calf has been investigated.
2. The sulphate content of the epiphyseal plate is little higher than that of hyaline cartilage.
3. The sulphate concentration of that area of cartilage where calcification begins is approximately the same as that of non-calcifying hyaline cartilage.
4. The precipitous fall in organically bound sulphate found at the visible front of calcification would seem to be the result of replacement of cartilage by osseous tissue.
5. There is no evidence from the present work to support the view that sulphated mucopolysaccharides disappear from cartilage before calcification occurs.

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