Calc. Tiss. Res. 3, 38—54 (1969)

Further Investigation on the Organic/Inorganic Relationships in Calcifying Cartilage*

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Received May 26, 1968

The organic/inorganic relationships in calcifying cartilage have been studied in tibial-femoral epiphyses of 5-day-old rats and in costochondral junctions of 1-month-old guinea pigs. The main results are:

(a) Apatite crystallites in areas of early cartilage calcification are easily removed from araldite and glycol methacrylate (GMA) sections by 1-hour treatment with 2% formic acid. (b) The decalcified areas have a very low electron density and do not contain recognizable structures. Only after osmium fixation can an amorphous material be found in them. (c) Independently from the fixative and the embedding medium, staining decalcified areas with uranyl acetate and/or lead citrate reveals thin, elongated structures (crystallite ghosts) similar in shape to apatite crystallites. (d) These crystallite ghosts and a dense background are stained by phosphotungstic acid (PTA) in GMA sections, a method which reveals polysaccharides. (e) The dense background is no longer stained by PTA after hyaluronidase digestion and the stainability of the crystallite ghosts is reduced. After uranyl acetate/lead citrate staining there are no recognizable changes due to hyaluronidase. But, on the other hand, the fine structure of the crystallite ghosts is modified by papain digestion. (f) Clusters of fibrillar material are present in the areas of early calcification when EDTA decalcification is carried out before embedding the specimens.

These results confirm the organic nature of the crystallite ghosts and suggest that they are formed by thin, rod-like protein/like structures (digested by papain) surrounded, and probably sheathed, by acid polysaccharides (digested by hyaluronidase and stained by PTA in GMA sections).

Key words: Epiphyseal cartilage — Calcification — Apatite crystals — Organic/Inorganic relationships.

Les rapports entre composants organiques et inorganiques du cartilage ont été étudiés au niveau des épiphyses tibiales chez des rats de 5 jours et au niveau des jonctions costo-chondrales chez des cobayes d'un mois.

Voici les principaux résultats auxquels on est parvenu: (a) Dans les zones où la calcification s'amorce les cristallites d'apatite sont susceptibles d'être facilement éliminés des coupes en araldite et en glycol methacrylate (GMA) après traitement pendant une heure par une solution d'acide formique au 2%. (b) Les zones décalcifiées sont douées d'une faible densité aux électrons et vont exemptes d'une structure quelconque. Seulement la fixation à l'osmium peut révéler du matériel amorphe. (c) Indépendemment du fixateur et du moyen d'inclusion, les coupes décalcifiées, traitées par l'acétate d'uranyl et/ou par le citrate de plomb, montrent de fines structures allongées dont l'aspect est semblable à celui des cristallites. (d) Ces structures ainsi qu'un fond dense interposé prennent l'acide phosphotungstique (PTA) dans les coupes en GMA, une méthode celle-ci qui met en évidence les polysaccharides. (e) Le fond dense ne prend plus le PTA après traitement par la hyaluronidase et en même temps les structures semblables au cristallites apparaissent plus faibles. Après traitement par l'acétate d'uranyl et le citrate de plomb, l'attaque par la hyaluronidase ne produit aucune modification appréciable. Au contraire l'aspect des structures semblables aux cristallites apparait modifié

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* This investigation was supported by a grant of the Italian Research Council.

par la digestion à la papaïne. (f) Quand la décalcification à l'EDTA précéde l'enrobage, des amas de matériel fibrillaire se mettent en évidence dans les zones où la calcification s'était amorcée.

L'ensemble de ces résultats permet d'établir la nature organique des structures semblables au cristallites et porte aussi à admettre que celles-ci soient formées par de fins filaments cylindriques (digérés par la papaïne) entourés, et probablement engainés par del polysaccharides (digérés par la hyaluronidase et prennant le PTA dans les coupes en GMA).

Die Wechselbeziehungen zwischen den organischen und inorganischen Substanzen des verkalkenden Knorpels wurden in der tibialen und femuralen Epiphyse 5 Tage alter Ratten und in den Rippen 1 Monat alter Meerschweinchen untersucht.

Die Hauptergebnisse waren:

a) Die Hydroxyapatitkristalle der Frühverkalkungszonen können in Araldit- wie auch in Glycolmethylacrilatschnitten (GMA) mit einer 1stündigen Behandlung durch 1%ige Ameisensäure leicht entfernt werden.

b) Die entkalkten Zonen haben eine sehr niedrige Elektronendichte und enthalten keine sichtbaren Festkörper. Nach Fixierung mit Osmiumsäure kann man darin ein formloses Material erkennen.

c) Eine Färbung der Schnitte mit Uranacetat und Bleicitrat, welche von den Fixier- und Einbettungsmitteln nicht beeinträchtigt wird, zeigt feine, längliche Körper („Kristallgeister") an, welche morphologisch den Kristallen von Hydroxyapatit gleichen.

d) Die Färbung der GMA-Schnitte mit Phosphowolframsäure (PWS), eine Methode welche Polysaccharide anzeigt, hob diese „Kristallgeister" besonders hervor und demonstrierte auch eine dichte dazwischenliegende Grundsubstanz.

e) Diese Substanz ist nach Behandlung mit Hyaluronidase nicht mehr färbbar mit PWS; die Färbbarkeit der „Kristallgeister" wird dabei jedoch nur verringert. Die Hyaluronidasebehandlung verändert die Uranacetat- und Bleicitratfärbung nicht. Die Feinstruktur der „Kristallgeister" wird durch Papainbehandlung sehr angegriffen.

f) Falls die Schnitte vor dem Einbetten mit EDTA entkalkt werden, können Faserbündel in den Frühzonen der Verkalkung nachgewiesen werden.

Diese Ergebnisse bestätigen die organische Natur der „Kristallgeister". Sie beweisen auch, daß dieselben aus feinen Proteinstäbchen bestehen (verdaulich in Papain) und von Polysacchariden umgeben und vielleicht auch überzogen sind (verdaulich in Hyaluronidase und anfärbbar mit PWS).

Introduction

Great interest has always been shown in the relationship between the organic and inorganic substance in calcified tissues, because deeper knowledge of this relationship may shed further light on the mechanism of calcification. It has been suggested that this mechanism may be the same in cartilage and in bone, even though in calcifying cartilage the crystallites are not aligned along the collagen fibrils in the same way as in bone (Robinson and Cameron, 1956; Fitton Jackson, 1960; Takuma, 1960), but in the former case lie primarily between the fibrils (Robinson and Cameron, 1956). This disorderly distribution of crystallites does not necessarily mean that the mechanism of calcification in cartilage is different from that in bone, because it may be due to the small size of the fibrils (Glimcher, 1959). However, since a heavy concentration of crystallites has been reported in cartilage areas containing very few, or no, collagen fibrils, a different mechanism of calcification from that suggested by Glimcher was considered possible (Cameron, 1963). This hypothesis is substantiated by the observation that the early deposition of calcium salts in epiphyseal cartilage involves not only collagen fibrils, but also mainly interfibrillary, osmiophilic, amorphous bodies

(BONUCCI, 1967). Moreover, crystallites of early cartilage calcification lie against a dense, amorphous background which may play some part in the mechanism of calcification. Staining with phosphotungstic acid completely removes the crystallites and reveals a "dense or solidified appearance" of the areas previously occupied by the mineral (TAKUMA, 1960). Using phosphotungstic acid, as TAKUMA did, and other decalcifying and staining techniques, BONUCCI (1967) showed that these dense areas contain elongated structures which have the same shape as the crystallites. There was evidence that these structures are organic and that there is a close relationship between them and the mineral, so they were called "crystallite ghosts". It was further suggested that these ghosts might be protein/polysaccharide structures.

Because of the important part crystallite ghosts might play in the mechanism of cartilage calcification, further investigation has been carried out to substantiate this conjecture using the same material as that used in previous research (BONUCCI, 1967), but with different methods of embedding and staining.

Material and Methods

Costochondral junctions of 1-month-old guinea pigs and tibial-femoral epiphyses of 5-day-old rats were dissected under ether anesthesia and reduced to small fragments. These were either fixed in 4% formalin, in 1% osmium tetroxide, or in 4% formalin followed by 1% osmium tetroxide, both buffered to pH 7.2 according to MILLONIG (1962). After acetone dehydration the specimens were embedded in araldite.

Formalin-fixed specimens were also embedded in glycolmethacrylate (GMA) according to LEDUC and BERNARD (1962, 1967). This water-soluble resin makes a great variety of histochemical procedures possible as well as enzymatic digestion, all carried out directly on the sections (LEDUC and BERNARD, 1962, 1967; MARINOZZI and BERNARD, 1963).

Ultrathin sections were examined under the electron microscope (Siemens Elmiskop 1 A) both unstained and also after being stained by one of the following methods: 2% uranyl acetate; lead citrate (REYNOLDS, 1963); 2% uranyl acetate and lead citrate; 1% phosphotungstic acid (PTA); 1% PTA for 1 min, followed by lead citrate for 10 sec.

Additional ultrathin sections from both araldite and GMA embedded specimens were left floating for 60 min on a 2% formic acid solution (pH about 1.5). After this treatment, sections are thorougly decalcified (BONUCCI, 1967). To verify this, 1-μ-thick sections were decalcified together with the ultrathin ones, and these latter were considered decalcified if the former were negative to the von Kossa method. Moreover, decalcification was also directly checked under the electron microscope on decalcified, unstained sections, since, of course, no crystals are left in decalcified areas. The same check was also made by electron diffraction of selected decalcified areas. Decalcified sections were examined by using one of the staining methods reported above.

Several procedures have been suggested to show up protein/polysaccharides under the electron microscope: colloidal iron (CURRAN *et al.*, 1965), alcian blue (TICE and BARRNETT, 1962; OHKURA, 1966), bismuth nitrate (SERAFINI-FRACASSINI and SMITH, 1966; SMITH *et al.*, 1967), ruthenium red (LUFT, 1964). Unfortunately, all these methods must be applied on unembedded tissue blocks, which make them unsuitable for studying crystallite ghosts, since these are partly solubilized if the decalcification is carried out on unembedded tissue (BONUCCI, 1967).

Recently, two methods have been suggested which can be used directly for ultrathin sections. Both are based on the use of water-miscible methacrylate (hydroxypropyl or glycol methacrylate) after "inert dehydration" (PEASE, 1966) or after aldehyde fixation (MARINOZZI, 1967); sections are stained with PTA in both cases. Marinozzi's method was used in the present investigation for sections from formalin-fixed, GMA-embedded specimens.

To show up neutral polysaccharides, decalcified sections were treated with silver-methenamine impregnation after periodic acid oxidation (MARINOZZI, 1961). The technique stains the

same structures as the periodic acid-Schiff method (PAS) and can also be considered specific for aldehydic groups (RAMBOURG, 1967). In order to verify this staining reaction, additional decalcified sections, some oxidized with periodic acid, and others not, were left to float at 60° for 30 min on the alkaline solution (pH about 8.7) used for silver nitrate and then stained with uranyl acetate and lead citrate.

Decalcified sections from GMA-embedded specimens were digested with hyaluronidase and with papain. Testicular hyaluronidase (Nutritional Biochemicals) was used in saline solution (1 mg/ml), for 2 h at 37°. Papain (crude product, Nutritional Biochemicals) was used in 0.02 N acetate buffer (1 mg/ml) with KCN and EDTA, pH 5.4, for 15 min at 37°. Control sections were left floating on the respective buffers at the same temperature and for the same lengths of time as the experimental sections. After having been carefully washed with distilled water, the extracted ultrathin sections and the controls were stained both with uranyl acetate/lead citrate and with PTA.

For all the methods of decalcification, staining, and enzymatic digestion, free-floating sections were used according to MARINOZZI (1961) in order to avoid metal contamination from the grids.

Small epiphyseal specimens were fixed in formalin and osmium tetroxide, decalcified for 48 h with EDTA at pH 7.0, and then embedded in araldite. The sections were stained with uranyl acetate/lead citrate and with PTA.

Results

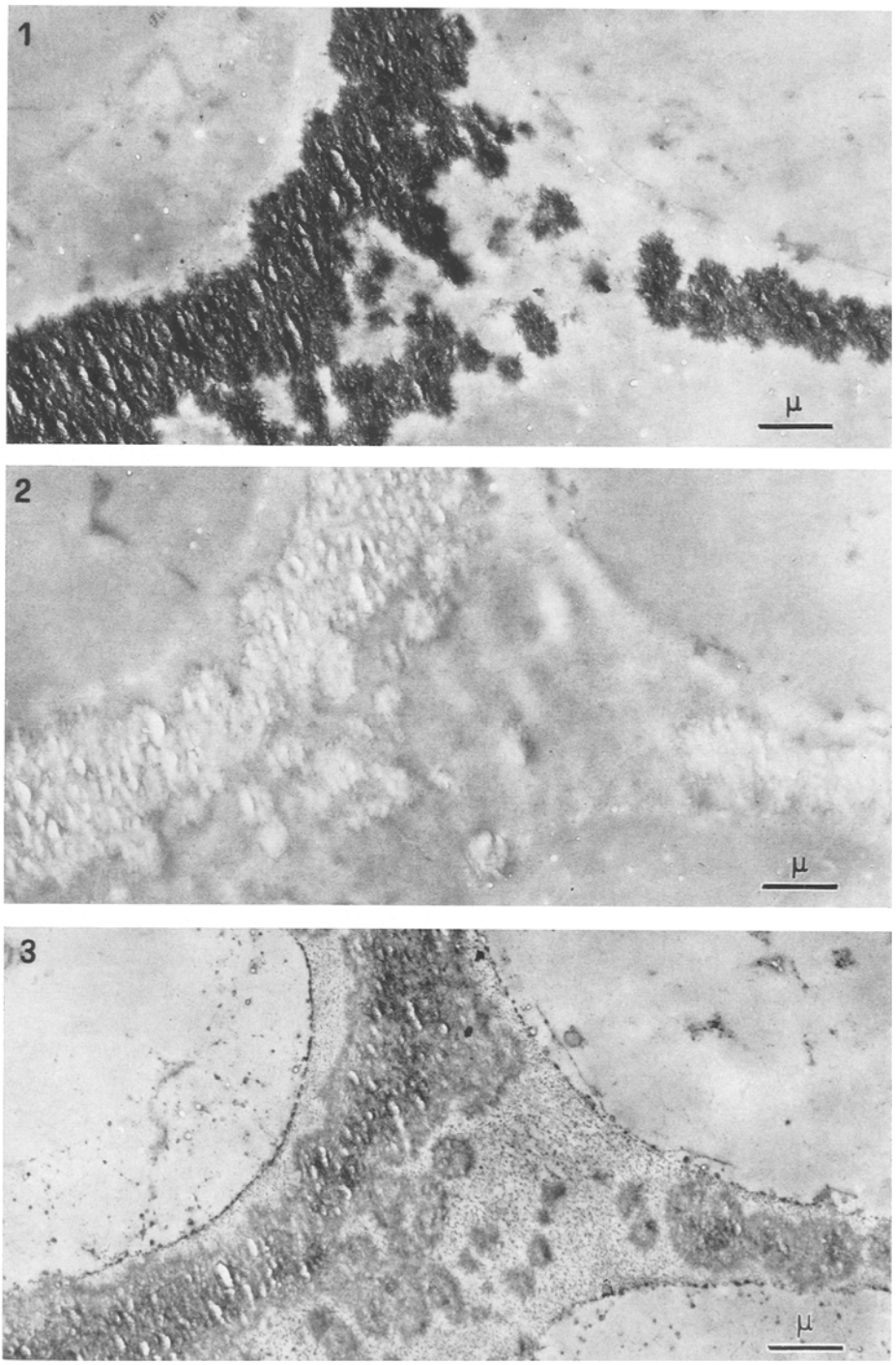
a) Untreated Sections. The fine structure of early cartilage calcification is now well known. The present investigation deals mainly with the zone of degenerating cartilage, in which isolated or coalescing clusters of apatite crystallites are recognizable (Fig. 1).

b) Decalcified Sections. When studying the organic matrix of the calcified areas it is necessary to ascertain that all the crystallites have been removed.

The best way of checking the degree of decalcification would seem to be to photograph the same area under the electron microscope before and after decalcification. Unfortunately, apatite crystals are no longer decalcifiable once exposed to the electron beam of the microscope. Likewise, organic structures are no longer stainable. Attempts were made to overcome this difficulty by means of ultrathin serial sections, some of which were examined without having undergone any treatment, while others were examined after formic acid decalcification, and the remainder after decalcification and staining. Three of these serial sections are shown in Figs. 1, 2, 3. It became clear that apatite crystallites were completely removed after formic acid treatment even down to the smallest crystal clusters, each decalcified cluster appearing as an amorphous area, the electron density of which was lower than that of the adjacent plastic (Fig. 2). At high enlargement and in sections from osmium-fixed specimens, these decalcified areas can be seen to contain amorphous material of low electron density, which is not recognizable in sections from formalin-fixed specimens. In this case the decalcified areas are almost transparent to the electrons. Of course, electron diffraction patterns from these areas do not contain apatite reflections.

The von Kossa method used on undecalcified thick sections reveals even the smallest areas of calcification. After decalcification, the von Kossa method is completely negative both in araldite and GMA sections, confirming the finding that the reacting mineral is completely removed.

c) Decalcified and Stained Sections. Staining decalcified sections makes it possible to examine the fine structure of the previously-calcified organic matrix.



Figs. 1—3

The results obtained by staining decalcified sections from araldite-embedded specimens with uranyl acetate and/or lead citrate as well as with PTA do not differ from those reported in a previous paper (BONUCCI, 1967). The decalcified areas, which are almost transparent to the electrons in unstained sections, are deeply stained (Fig. 3) and contain elongated structures (about 1,500 Å long) situated inside an amorphous substance of low electron-density (Fig. 4). Although thicker than untreated apatite crystallites (about 35 Å as against 20 Å), these structures have the same needle-shaped appearance as the crystals and the same arrangement in clusters. Owing to this similarity they were called "crystallite ghosts" (BONUCCI, 1967), and this term will be used here.

PTA/lead citrate staining does not greatly change these findings, except that the electron contrast of the ghosts is increased. On the other hand, crystallite ghosts are not recognizable in ultrathin sections treated with silver nitrate-methenamine. Aggregates of small silver granules are alone present in previously calcified areas. After periodic-acid oxidation these granules disappear, the areas previously occupied by the crystal clusters now appearing as areas of very low electron density surrounded by deeply impregnated collagen fibrils (Fig. 5). This negativity of the silver-methenamine method is not due to loss of the crystallite ghosts, since these are again clearly visible if decalcified sections — both the ones oxidized with periodic acid and unoxidized ones — are treated in the same way as in the case of the silver nitrate-methenamine method, but with the silver nitrate omitted, and are then stained with uranyl acetate and lead citrate (Fig. 6).

In decalcified sections from formalin-fixed, GMA-embedded specimens, uranyl acetate and/or lead citrate reveal crystallite ghosts as above (Fig. 7). However, they are thicker (mean value 45 Å) than those found in araldite sections (mean value 35 Å). The electron contrast is less sharp than in araldite. In areas of advanced calcification, crystallite ghosts are present, but they are more clearly recognizable at the edge of these areas than in the centre. After 1% PTA staining, crystallite ghosts are still visible, but they are situated against an amorphous, electron-dense background and are partially masked by it (Fig. 8). In the areas of advanced calcification, this background completely masks the crystallite ghosts. After overstaining these PTA stained sections for 10 sec with lead citrate, the density of this amorphous material becomes such that it masks all other structures.

d) Decalcified and Extracted Sections. Hyaluronidase digestion of GMA-decalcified sections does not change the fine structure of the crystallite ghosts

Fig. 1. First of three serial sections (see Figs. 2 and 3) from a formalin/osmium-fixed, araldite-embedded specimen, showing a calcifying trabecula and three cellular lacunae. Crystallites are collected in isolated or coalesced clusters. Unstained, × 10,000

Fig. 2. Section treated for 60 min with 2% formic acid and unstained (see also Figs. 1 and 3). The decalcified areas have a lower electron density than the adjacent regions, × 10,000

Fig. 3. Section treated for 60 min with 2% formic acid and stained with uranyl acetate and lead citrate (see also Figs. 1 and 2). The decalcified areas appear denser than the adjacent matrix. At higher enlargement crystallite ghosts are clearly visible (see Fig. 4), × 10,000

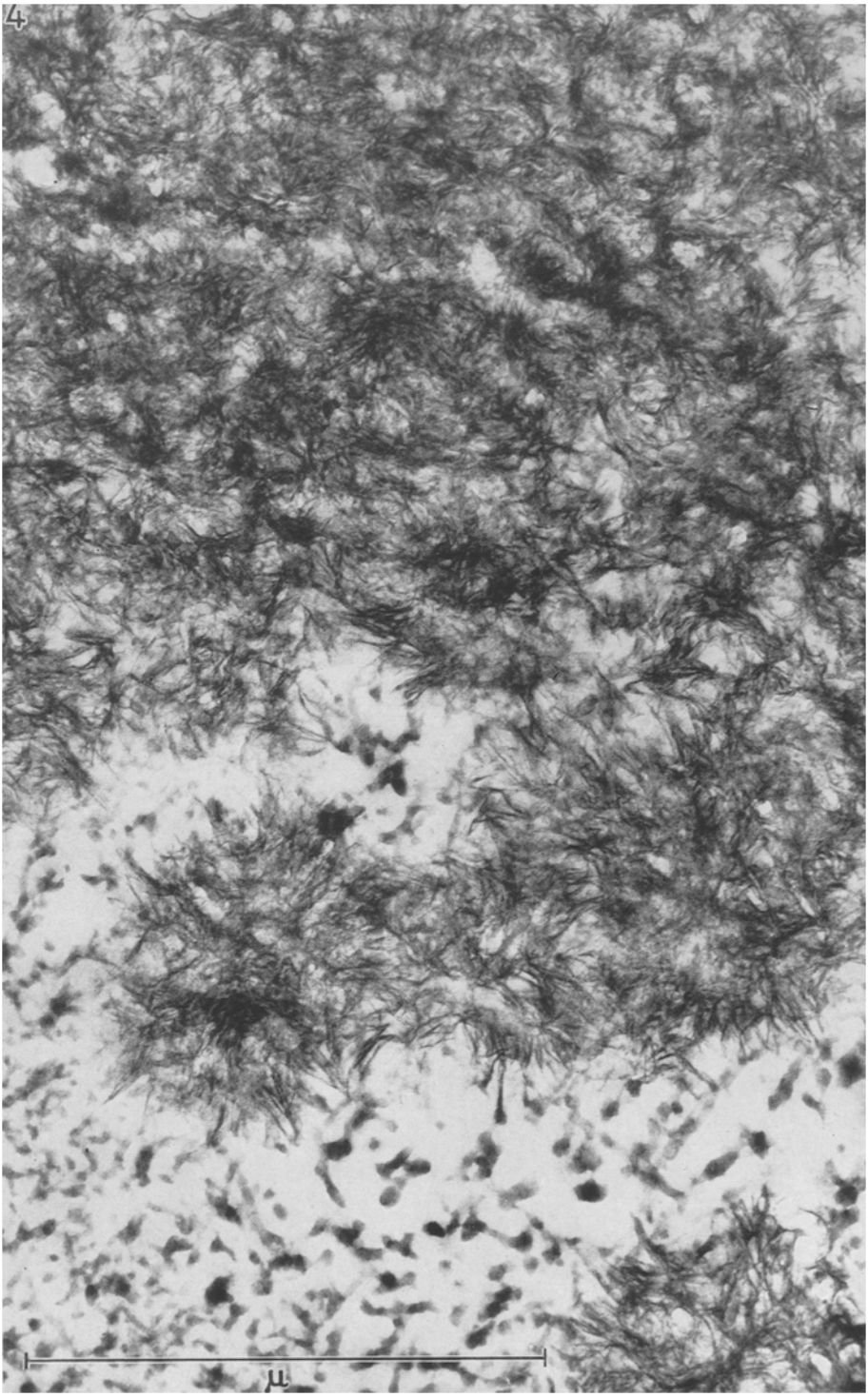


Fig. 4

stained with uranyl acetate and lead citrate. The ghosts, however, are more deeply stained than in controls.

If the digested sections are stained with PTA, the crystallite ghosts are hardly recognizable and the amorphous background against which they lay in unextracted sections is practically unrecognizable.

As regards papain digestion, some changes in the fine structure of the crystallite ghosts also occur in control sections stained with uranyl acetate/lead citrate. Crystallite ghosts are easily recognizable in these sections, but they are less stained and show a lower electron contrast than the same structures found in unextracted sections (Figs. 9, 11). These changes are actually much clearer in experimental sections, where crystallite ghosts are either unrecognizable (Fig. 12), or are swollen, lightly stained and only just recognizable (Fig. 10). After PTA staining there are no differences between experimental and control sections.

e) EDTA Decalcification before Embedding. Apatite crystallites are of course not visible in these sections. However, the areas of early calcification are easily recognizable, thanks to clusters of thin fibrils which are almost radially arranged (Fig. 13). Some of these fibrils are very thin, almost as thin as the crystallite ghosts described above. When cut tangentially, they appear as small, electron-dense dots. No difference was observed between uranyl acetate/lead citrate and PTA stained sections.

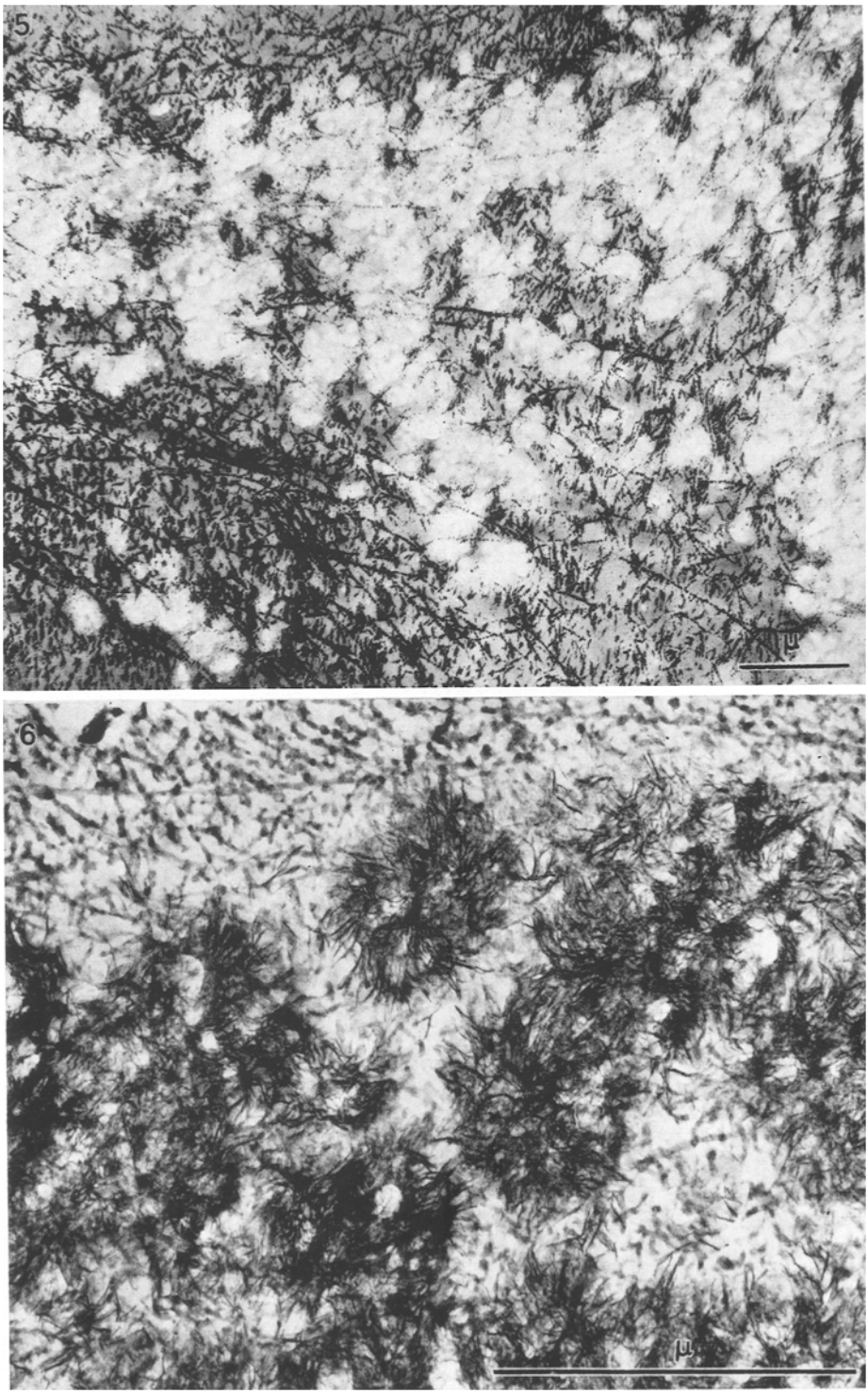
Discussion

This investigation on early cartilage calcification shows above all that the organic matrix of the calcified areas is formed not only by thin collagen fibrils, but also by very thin, elongated structures (the crystallite ghosts), which are similar in shape to untreated crystallites and which are surrounded by a dense, amorphous background.

The similarity between the crystallite ghosts and untreated crystals might be taken as meaning that the ghosts are actually undecalcified crystallites left in partially decalcified sections. However, it is easy to demonstrate that the ghosts are organic structures.

Firstly, they are recognizable only after staining. Moreover, the von Kossa method, as well as direct examination under the electron microscope of undecalcified unstained sections, and electron diffraction, all show that the mineral is completely removed from the ultrathin sections after treatment for one hour with formic acid. This is particularly clear in serial sections. Furthermore, crystallite ghosts are not revealed by the silver nitrate-methenamine method in decalcified sections, while they are clearly visible if these sections are stained with uranyl acetate/lead citrate. These results demonstrate beyond all doubt that the crystallite ghosts are not apatite crystallites left in the decalcified sections, since, of course, in this case they would always be visible, independently of the staining

Fig. 4. Section from a formalin-fixed, araldite-embedded specimen, treated for 1 h with 2% formic acid and then stained with uranyl acetate and lead citrate. Crystallite ghosts are clearly visible. They show the same arrangement in isolated or coalesced clusters as apatite crystallites in untreated sections, × 72,000



Figs. 5 and 6

methods used. On the other hand, the organic nature of the crystallite ghosts is also shown by the enzymatic digestion with papain, as discussed below. Finally, the great permeability of GMA must also be considered. If the large molecules of the enzymes can penetrate this plastic, there is no reason to deny its permeability to formic acid. It must be borne in mind that decalcification begins in about 5 min if araldite sections are left to float in distilled water whose pH is about 5.5 (BOOTHROYD, 1964), while in the present investigation the ultrathin sections were left for 60 min on a strong acid solution (pH 1.5).

If decalcification is complete, and if no crystallites are left in the sections, the conclusion must be drawn that the crystallite ghosts are organic. Moreover, since they have the same shape as the untreated crystallites, and since it is impossible to reveal them before removing the mineral, it must be deduced that each ghost takes part in the formation of a crystallite (see also BONUCCI, 1967).

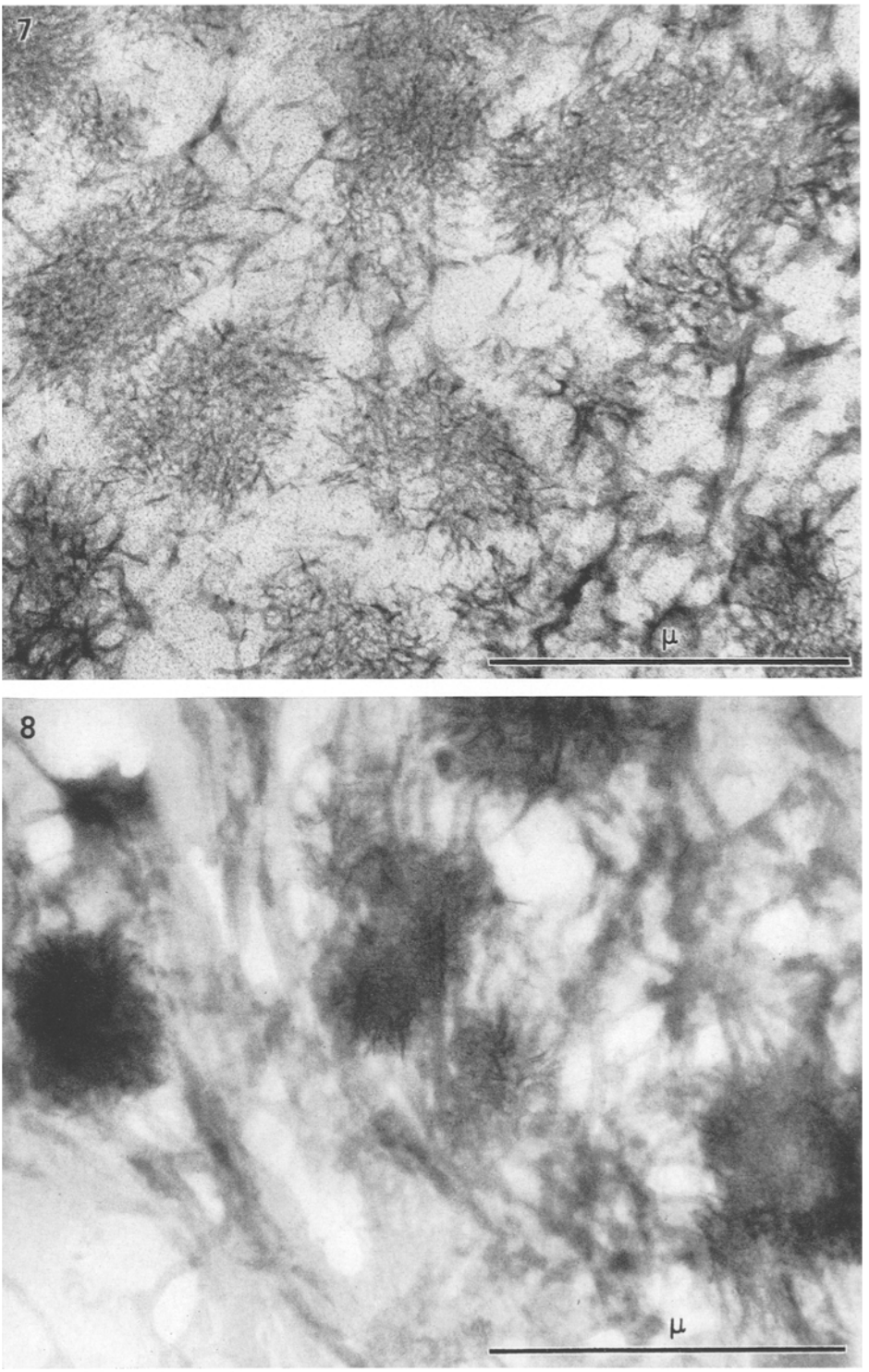
Sections from specimens decalcified before embedding contain clusters of thin fibrils, sometimes similar to the crystallite ghosts, in the previously calcifying areas. Although ghosts are not recognizable, perhaps because of an EDTA-solubilizing effect, the presence of these finely fibrillary clusters is additional evidence that a particular organic substrate is present in the areas of early calcification.

Nucleation of calcium salts by organic substrate different from collagen is now admitted as a possibility in a variety of tissues (GLIMCHER, 1959; PAUTARD, 1965, 1966; SOBEL, 1965; HÖHLING and VAHL, 1966; ENNEVER and CREAMER, 1967) and perhaps the best demonstration of this possibility is the calcification of enamel (FRANK *et al.*, 1960; RÖNNHOLM, 1962; TRAVIS and GLIMCHER, 1964; GLIMCHER *et al.*, 1965; BONAR *et al.*, 1965). As regards the cartilage, it has been suggested that the process of calcification is similar to that in bone, that is, in both these tissues calcium salts would seem to be nucleated by collagen fibrils (GLIMCHER, 1959). However, morphological evidence showing that crystallites may be unrelated to collagen fibrils (CAMERON, 1963), and that they can also be nucleated in interfibrillary osmiophilic bodies (BONUCCI, 1967), suggests that some other nucleating mechanism is present. Evidence that an organic framework (the crystallite ghost) takes part in the formation of the crystallites suggests that it may nucleate calcium salts in the cartilage.

This organic framework, or ghost, does not react with silver nitrate-methenamine; aggregates of silver granules are alone present in the previously calcified areas. These granules are probably due to the precipitation of silver on reduced osmium present in the organic matrix (see MARINOZZI, 1961). They disappear after periodic acid oxidation, since the osmium is removed by this treatment. The

Fig. 5. Section from a formalin/osmium-fixed, araldite-embedded specimen, decalcified with formic acid, treated with 1% periodic acid, left for 30 min at 60° in the same borax-menthenamine solution used for the silver nitrate method, and then stained with uranyl acetate/lead citrate. Clusters of crystallite ghosts are visible (c. f. Fig. 6), × 50,000

Fig. 6. Section from a formalin/osmium-fixed, araldite-embedded specimen, decalcified with formic acid, treated with 1% periodic acid, and stained with silver nitrate-menthenamine. Only the collagen fibrils of the matrix are stained. The previously calcified areas appear to be empty and show a very low electron/density. Neither crystallites nor crystallite ghosts are visible, × 15,000



Figs. 7 and 8

previously calcified areas appear empty and the crystallite ghosts are unrecognizable. The negativity of the silver nitrate-methenamine method after periodic acid oxidation would seem to prove that no neutral polysaccharides are present in the matrix of the calcified areas. However, this is in contradiction to histochemical findings showing that the calcified cartilage is PAS-positive (see Pritchard, 1952; Cabrini, 1961). This PAS positivity is also evident in thick sections from araldite-embedded specimens. At the moment the different results obtained with periodic acid-Schiff and periodic acid-silver nitrate in calcifying cartilage defy explanation.

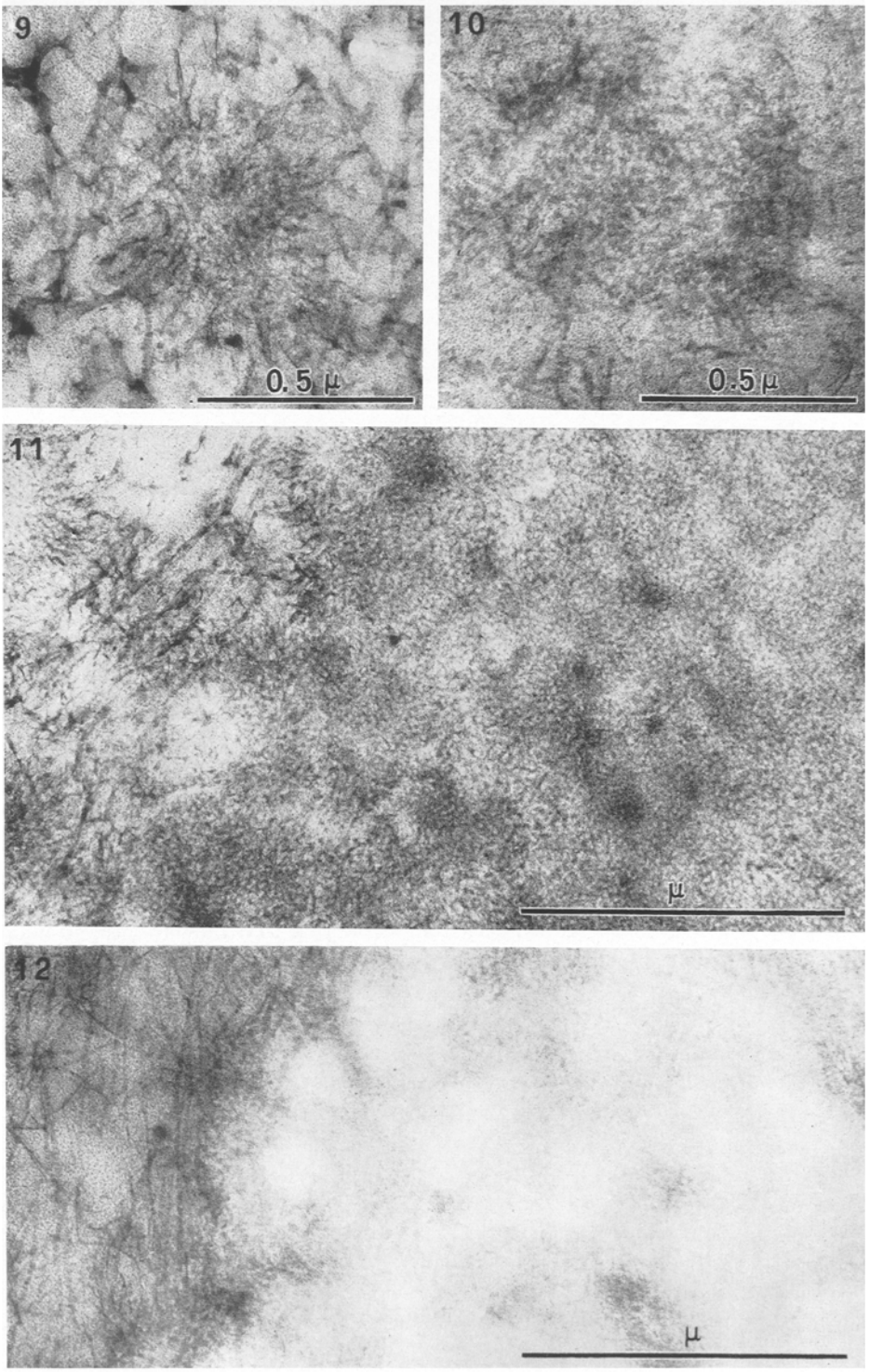
PTA staining of GMA sections reveals not only the crystallite ghosts, but the dense background against which they lay. Since PTA shows polysaccharides in GMA sections (Pease, 1966; Marinozzi, 1967), both the crystallite ghosts and the background might be considered as polysaccharides. However, PTA staining disappears in the background after hyaluronidase digestion, while it only decreases in crystallite ghosts. Moreover, the fine structures of the crystallite ghosts is unchanged if the hyaluronidase-digested sections are stained with uranyl acetate/lead citrate. These results prove that the background is formed by hyaluronidase-degradable polysaccharides, that is, acid polysaccharides containing chondroitin sulphate. This is in agreement with the well-known fact that the decalcified cartilage stains metachromatically with basic dyes and that ^{35}S rapidly accumulates in the matrix of calcifying cartilage (see Weidmann, 1963). On the other hand, the crystallite ghosts seem to be formed by another protein-like/substance too, or by that substance alone. This is confirmed by papain digestion which deeply changes the fine structure of the crystallite ghosts. Although some change also occurs in control sections, it seems justified to believe the crystallite ghosts are digested by papain and, consequently, proteinaceous/structures.

It is difficult to obtain histochemical information from techniques available at present for electron microscopy, chiefly owing to interference of the plastic in which the tissue is embedded. However, the present results, besides showing that the crystallite ghosts are organic, also seem to indicate that they are protein-like, since they are altered by papain digestion, and that they lay against a background containing acid polysaccharides. In conclusion, the crystallite ghosts seem to be rod-like/proteinaceous structures surrounded, and probably sheathed, by acid polysaccharides.

The structure of the cartilage protein/polysaccharide complexes is still uncertain, but the proposed model corresponds to a rod-like structure, formed by a proteic core to which lateral chains of chondroitin sulphate are attached (Mathews and Lozaityte, 1958; Partridge *et al.*, 1961; Cessi and Bernardi, 1965). The

Fig. 7. Section from a formalin-fixed, GMA-embedded specimen, decalcified with formic acid and stained with uranyl acetate/lead citrate. Clusters of crystallite ghosts are visible. These are thicker and show a lower contrast than those found in araldite sections, × 50,000

Fig. 8. Sections from a formalin-fixed, GMA-embedded specimen, decalcified with formic acid and stained with PTA. Clusters of crystallite ghosts are visible. These are partially masked by the amorphous background, × 50,000



Figs. 9—12

crystallite ghosts are very similar to this model. Although they may simply be protein filaments in a polysaccharide background, it may also be conjectured that they are true proteinpolysaccharide complexes.

Of course, this is only a conjecture, but it would seem to agree with the evidence that the protein core of protein/polysaccharide complexes is not degraded by hyaluronidase, which only splits off the polysaccharide chains, while it is promptly digested by papain (MATHEWS and LOZAITYTE, 1958; MUIR, 1958, 1964; PARTRIDGE *et al.*, 1961). This behaviour is similar to that of the crystallite ghosts. Moreover, the thickness (from 30 to 45 Å) and the length (about 1,500 Å) of the crystallite ghosts are similar to those of the basic molecular unit proposed by MATHEWS and LOZAITYTE (1958; see also MATHEWS, 1965) for protein/polysaccharide complexes and calculated on the basis of the values of light scattering, viscosity and molecular weight (36 Å in thickness and 3,700 Å in length). They are also similar to the value (30 Å in thickness and from 1,100 to 1,500 Å in length) obtained by SERAFINI-FRACASSINI and SMITH (1966) for protein/polysaccharide complexes stained with bismuth nitrate and obtained from the light fraction of bovine nasal cartilage.

To pursue this speculation, it must also be borne in mind that in calcified areas the organic structures being "embedded" by the mineral may be protected against solubilization or morphological changes during fixation, dehydration, and embedding. This might explain why the protein/polysaccharide complexes, which of course are also present in the uncalcified matrix, would have a rod-like appearance only in calcified areas.

The importance of the polysaccharides in calcification has long been debated, and either a positive or negative role has been ascribed to them (see CAMERON, 1963; WEIDMANN, 1963). Recently, it has been shown that the protein/polysaccharide complex, or its protein component alone, may be lost or drastically altered during, or just preceding, calcification (HIRSCHMAN and DZIEWIATKOWSKI, 1966). A drastic alteration of the complex might well be explained by the fact that its protein core takes part in the formation of the crystallites.

Of course, the possibility must also be considered that crystal ghost are non-polysaccharidic proteinaceous filaments. Keratin-like material, such as that described in enamel and baleen (see PAUTARD, 1966) might chiefly be considered.

The technical assistance of Mr. LUCIANO DI BALDO is gratefully acknowledged.

Fig. 9. A cluster of crystallite ghosts in a section used to verify papain digestion. Uranyl acetate/lead citrate, × 60,000

Fig. 10. A cluster of crystallite ghosts after papain digestion. The ghosts are swollen and seem fragmented (c. f. Fig. 9). Uranyl acetate/lead citrate, × 60,000

Fig. 11. Coalesced clusters of crystallite ghosts in a section used to verify papain digestion. Uranyl acetate/lead citrate, × 45,000

Fig. 12. Area of calcification in which coalesced clusters of crystallite ghosts were present after papain digestion. The crystallite ghosts are no longer recognizable and the previously calcified area appears almost empty (c. f. Fig. 11). Uranyl acetate/lead citrate, × 45,000

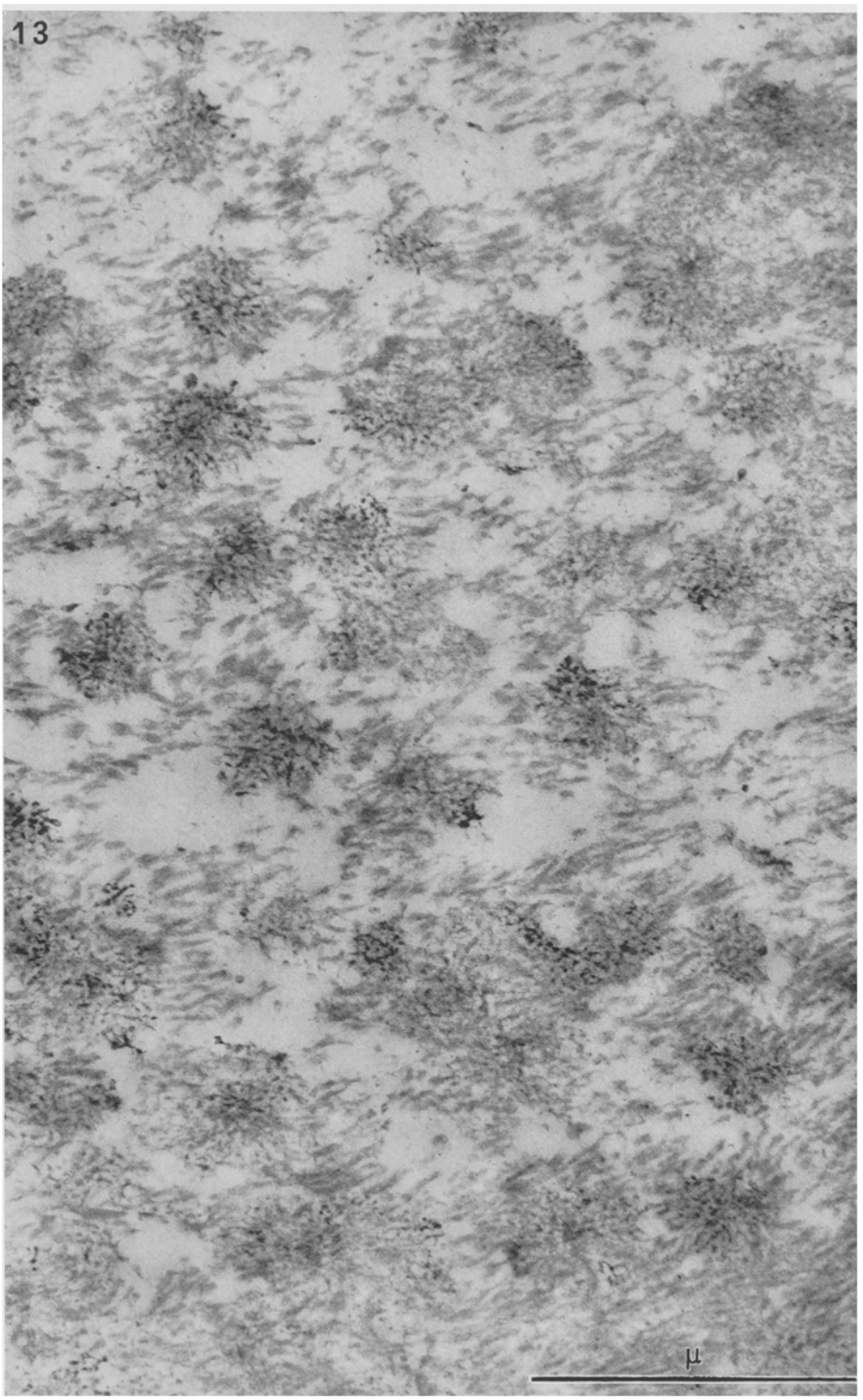


Fig. 13. Clusters of filamentous structures in a section from a specimen decalcified with EDTA before embedding. Uranyl acetate/lead citrate, × 45,000

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