BONE SALTS IN UNICELLULAR ORGANISMS

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In animals, calcium phosphate as hydroxyapatite[1] with occluded carbonates and citrates is usually confined to the mineral complement of bone and teeth in the Vertebrata. An apparent close X-ray diffraction relationship between the bone salts and the adjacent collagen fibrils has been found by CARLSTRÖM, ENGSTRÖM AND FINEAN[2], but there is as yet no clear-cut evidence on the mode of transport of the participating ions or on the growth and alignment of bone crystallites. FITTON-JACKSON AND RANDALL[3], however, have demonstrated the presence of small particles in embryonic skeletal tissue.

In the Invertebrata, calcification predominantly involves the carbonate, which varies from amorphous deposits to well-characterized crystalline calcite or aragonite[4]. Calcium phosphate is largely absent from skeletal structures, although POBEGUIN has recently suggested that phosphate may stabilize the colloidal carbonate of certain crustaceans[5].

During the course of work on the structure of flagella and other motile organs it has been observed that culture of unicellular flagellated algae in media containing proprietary casein hydrolysate preparations (Difco "bacto-tryptone") and tapwater has resulted in flagella giving X-ray diffraction diagrams closely resembling those of hydroxyapatite. While a note of caution has already been sounded about these artifacts[6], substances also closely resembling bone salts have now been noted in the ciliate *Spirostomum ambiguum* collected from natural sources or grown in a medium free of "bacto-tryptone", and it would therefore appear that the power of formation of such inorganic complexes may be inherent in the metabolism of these unicellular creatures.

This paper is a preliminary account of the appearance of hydroxyapatite both in cultured and in naturally-occurring organisms.

EXPERIMENTAL

The flagellated unicellular algae: *Polytoma uvella*, *Polytomella caeca* and *Chlorogonium elongatum*, and the ciliate *Spirostomum ambiguum* were obtained from the Culture Collection of Algae and Protozoa, Cambridge. Naturally-occurring *Spirostomum* was collected from a long-established pond at Bramhope, near Leeds.

The algae were grown in sterile neutral media containing combinations of sodium acetate (0.2%), yeast extract (0.2%), Difco "bacto-tryptone" (0.2%) in distilled or tap water. *Spirostomum* was allowed to thrive in a tank containing source pondwater (pH 6.8) with weeds, mud and organisms from the pond, or cultured in a barley–soil–tapwater–chalk medium containing yeast extract but no bacto-tryptone.

After growth of algae in acetate media (10–14 days) the pH of the exhausted culture fluid was generally in the region of 9.0 and the unfixed cells were harvested by centrifugation at 1500 × *g*. They were then washed once or twice by careful suspension in about 50 vol. distilled water followed by centrifugation, again at 1500 × *g*. The washed algae (about 60% of the flagella

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were still attached to the bodies at this stage) were suspended in 20 vol. distilled water and the flagella detached from the cells by sharp shaking for about 15 sec. After separation from the cell bodies by centrifugation at 3000 × *g* the suspended flagella were sedimented to a viscid pellet by further spinning at 8000 × *g*. For X-ray examination the flagella were taken up in a small (about × 2) volume of distilled water and then dried down on a glass block treated with a water-repellent (dichlordimethylsilane or a suitable silicone). When quantities permitted, the films of flagella were cut into narrow (1 mm) strips which were mounted on top of one another across a slotted brass support. More usually, the dried flagella were scraped from the glass block and heaped into a narrow fillet, after wetting, on a very thin collodion film (prepared by allowing a drop of collodion in amyl acetate to spread over a water surface) mounted across a brass support.

Spirostomum ambiguum cells were prepared for diffraction analysis by first washing the animals in distilled water and then mounting them on a thin collodion film on a brass support by transferring them one at a time on a fine glass fibre, allowing in this way up to 200 organisms to dry "en masse" in a drop about 1 mm in diameter.

All specimens were examined in standard diffraction equipment using CuKα radiation collimated by ½-mm lead-glass capillary, and a flat-film camera with a 2 cm or 4 cm specimen-to-film distance.

RESULTS

Normal flagella pattern in algae

The flagella of *Polytoma*, *Polytomella*, and *Chlorogonium* separated from cells grown in a medium containing acetate–yeast extract–bacto-tryptone in *distilled* water show in the electron microscope the now familiar arrangement of two inner, and nine outer, subfibrils. Their X-ray diffraction pattern indicates a protein, but unoriented and not sufficiently specific in these diagrams to establish the presence of either the α and/or β configurations. This "normal" pattern, shown for *Polytoma* flagella in Fig. 1, is characteristic of all the algal flagella studied (including the marine alga *Syracosphaera* growing naturally in sea-water) and of the flagella of the spermatozoa of fish such as *Perca fluviatilis*, *Salmo fario*, *Salvelinus willoughbii*, *Gadus morrhua*, *Esox lucius* and *Harengus harengus*.

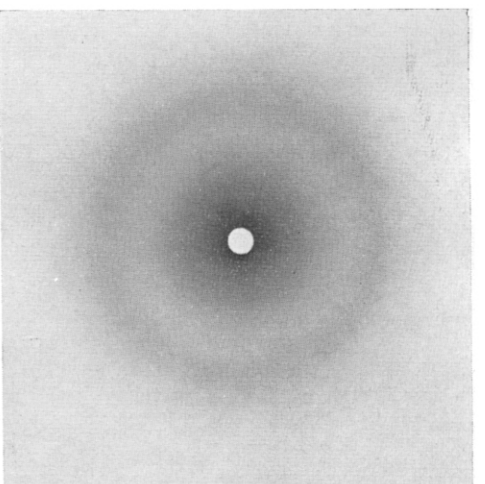

Fig. 1. Flagella from *Polytoma uvella* grown in sodium acetate (0.2 %), yeast extract (0.2 %) and bacto-tryptone (0.2 %) in distilled water. Powder diagram of dry flagella on collodion film.

"Apatite" patterns in algae

When *Polytoma*, *Polytomella* and *Chlorogonium* were grown in media containing bacto-tryptone and *tapwater* as the two common factors, the flagella appeared to become encrusted with many electron-dense flat plates, often to such an extent that the subfibrils were barely discernible. These deposits, visible in and near the *Polytoma*

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flagellum shown in the electron micrograph in Fig. 2, vary from particles less than about 50 Å in diameter to plates measuring about 6000 Å × 2000 Å, depending on the age and composition of the culture. The X-ray diffraction pattern (Fig. 3) of these encrusted flagella reveals the presence of low-molecular-weight substances, the main reflexions agreeing very closely with those of defatted ox vertebral bone (shown in the comparison photograph in Fig. 3). The algal cell bodies appeared not to contain these apatite-like substances and the major bulk of the artifact seemed to be concentrated on the flagella.

When treated with 0.1 *N* NH_4OH, an aqueous solution of bacto-tryptone gave a precipitate, from which a lighter, more colloidal, fraction was separated by centrifugation. This fraction, when dried and mounted, gave an X-ray diffraction pattern similar in many respects to the algal "apatite" pattern. Moreover, algae grown in a *tapwater* medium containing yeast extract, acetate and the supernatant after treating bacto-tryptone with ammonia, gave a "normal" diffraction pattern devoid of any "apatite" spacings.

Flagella showing apatite artifacts were decalcified in 1–4 *N* HCl. The residual material gave pronounced diffraction patterns suggesting protein but the size of the preparations was too small to test for collagen with any certainty. Autoclaving the flagellar "bone" in distilled water resulted in a soluble fraction, also too small to be satisfactorily examined.

Spirostomum ambiguum

All the specimens of *S. ambiguum* examined so far have shown evidence of hydroxyapatite, although this has not yet been identified with any specific part of the organism. A typical "bone salt" pattern from *Spirostomum* is shown in comparison with ox vertebral bone in Fig. 4, and as in the case of the flagella artifacts there are strong reflexions in the region of 4.65 Å and 9.8 Å indicative of protein. These and the extensive low-angle scatter tend to obscure some of the detail, but the reflexions that are visible, are in very close agreement with those from bone.

Comparison of reflexions

A comparison of reflexions from flagellar and *Spirostomum* hydroxyapatite and ox vertebral bone is shown in Table I. The characteristic strong reflexions at 2.8 Å and 3.4 Å are present in every case, and the minor variations in the spacings of the weak and very weak reflexions are in keeping with the accepted lattice variations in the biological apatites (see CARLSTRÖM, for example).

DISCUSSION

Hydroxyapatite being the most stable form of calcium phosphate in the aqueous phase[8], its formation by precipitation of soluble calcium and phosphorus from slightly alkaline solution might therefore be expected during the growth of the acetate flagellates when the pH rises to 9 and more, and in fact the *in vitro* precipitation of apatite-like fractions from weakly alkaline solutions of bacto-tryptone in distilled water suggests that the bacto-tryptone is merely a convenient source of calcium and phosphate. Yet no apatite-like substances can be detected in *Polytoma* cells unless tapwater is present, and *in vivo* it would seem that some contribution from the tapwater salts is essential.

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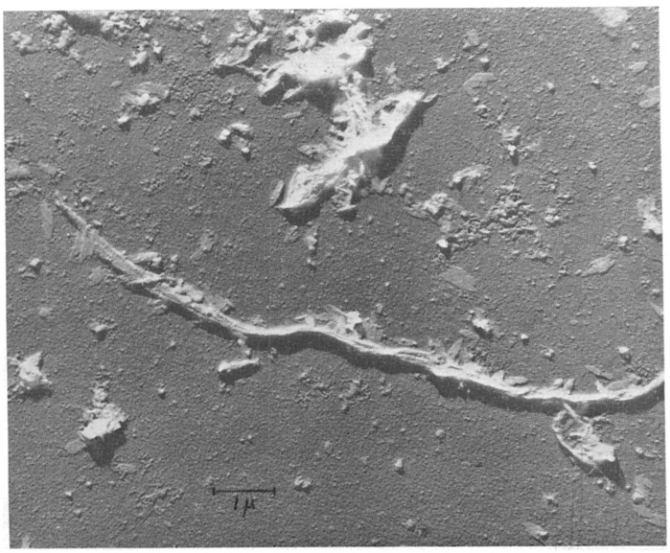

Fig. 2. Flagella from *Polytoma uvella* grown in sodium acetate (0.2%), yeast extract (0.2%) and bacto-tryptone (0.2%) in tapwater. Gold-shadowed. Numbers of platelets have become detached from the flagella during drying. The ill-defined dense masses represent the tangled and clumped flagella more usually observed.

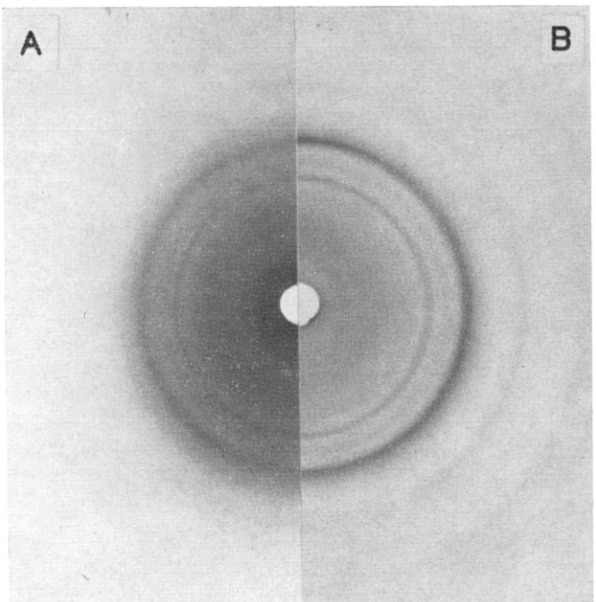

Fig. 3. Comparison between diffraction patterns from encrusted flagella (left-hand diagram A) and defatted ox vertebral bone (B). The flagella (dry powder on collodion film) were from *Polytoma uvella* grown in sodium acetate (0.2%), yeast extract (0.2%) and bacto-tryptone in tapwater. The bone, as filed dust, was also mounted on collodion film. Original comparison: 2 cm specimen-to-film-distance.

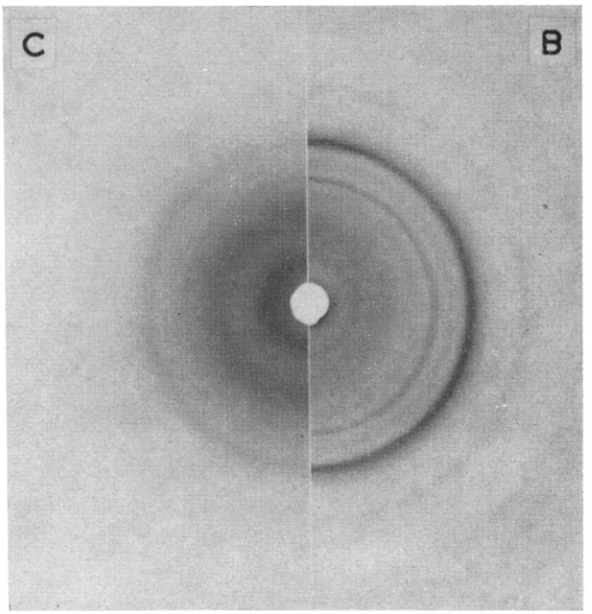

Fig. 4. Comparison between diffraction patterns from *Spirostomum ambiguum* (left-hand diagram C) and defatted ox vertebral bone (B). The *Spirostomum* preparation consisted of about 200 whole, dried animals (from soil–barley–chalk medium) mounted on collodion film. Original comparison: 2 cm specimen-to-film distance.

TABLE I

	Algal flagellar "hydroxyapatite" Å	*Spirostomum "hydroxyapatite"* Å	*Ox vertebral bone* Å
Chlorogonium	?	?	9.29 M
	6.55 V.W	?	6.30 W
	5.10 W	5.10 W	5.10 W
	4.07 V.W	3.67 V.W	3.86 V.W
	3.38 S	3.45 S	3.45 S
	3.10 M	3.09 M	3.12 M
	2.79 S	2.81 S	2.81 S
	2.54 W	2.64 W	2.64 W
	?	2.23 W	2.25 W
	?	1.93 V.W	1.93 W
	?	1.82 V.W	1.82 W

Again, the well-defined "crystalline" structures, found at the same time as X-ray evidence for hydroxyapatite, are seen on the flagella only and not (as might be expected) on the cell bodies also or in the culture solution. This suggests that the flagellum alone may be concerned with the precipitation of insoluble phosphate, perhaps as the site of some metabolic reaction-"fixing" and otherwise labile transfer of ions. It might be, for example, that since the locomotive activity of the flagellum may be influenced by the concentration of Ca^{++} and OH^- ions[9], the presence of phosphatases in the organ[10] might create high local concentrations of phosphate under conditions where attendant increase of pH allows irreversible precipitation of insoluble

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phosphate, particularly if the reaction is "fixed" by calcium carbonate or bicarbonate in the surrounding solution. Whatever the reason for the appearance of hydroxyapatite on the flagella of cells grown in tapwater–bacto-tryptone combinations, the way in which the plate-like encrustations build up on the flagellum might represent some, or all, of the calcifying mechanism that evolved the vertebrate skeleton, though whether, as in the case of bone, collagen (or some protein doing duty for collagen) is present as a ground substance, is not at present known. In spite of the apparent specificity of the bacto-tryptone, it is difficult to avoid a comparison between the flagellar artifact and the "artifact" of pathological ossification.

The presence of bone salt in *Spirostomum ambiguum* cannot, on the other hand, be directly attributed to an artifact, since no bacto-tryptone is present in the culture medium and the pH is near neutrality. The X-ray diffraction pattern of the whole animal, exclusive of the strong reflexions indicating protein, closely resembles that from bone, and in the circumstances it seems probable that the organism possesses an inherent mechanism for the precipitation of hydroxyapatite. The reason for the appearence of a calcium phosphate complex in so simple a creature is obscure, and especially so when it is remembered that the usual skeletal salt in the Invertebrata is calcite, aragonite or vaterite, often associated with a specific scleroprotein[11], but it may well be that this departure has some significance quite fundamental for the evolution of the vertebrate skeleton. Hydroxyapatite is usually closely associated with collagen in biological tissues and at present collagen has not been described in Phyla below the Coelenterata, but here at least is the suggestion that down in the Protozoa also there may be a collagen-like ground substance, capable moreover, under the right conditions, of forming bone by processes analogous to those present in higher animals.

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SUMMARY

1. X-ray diffraction diagrams of the flagella of *Polytoma uvella*, *Polytomella caeca* and *Chlorogonium elongatum* grown in a medium containing bacto-tryptone and distilled water indicate simply the presence of protein, but when these algae are grown in a medium containing bacto-tryptone and tapwater, there appear also apatite-like substances like those found in bone. Electron micrographs show that the flagella are at the same time encrusted with many flat electron-dense platelets.

2. Apatite-like precipitates have been obtained by the weakly-alkaline treatment of bacto-tryptone in distilled water.

3. X-ray diffraction diagrams of the ciliate *Spirostomum ambiguum*, collected from natural sources or grown in media free from bacto-tryptone, have also shown reflexions closely resembling those from bone.

4. The suggestion is put forward that in much lower organisms than has hitherto been suspected there may be a collagen precursor and an inherent power, given the right conditions, of forming bone-type hydroxyapatite by processes analogous to those found in higher animals.

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SOME COMPARATIVE OBSERVATIONS ON THE ELECTROSTATIC AND HYDRODYNAMIC BEHAVIOR OF BOVINE, HUMAN AND MONKEY GROWTH HORMONES*

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Recent investigations have disclosed some interesting similarities between growth hormones isolated in pure form from human and monkey pituitary glands[1]; the corresponding bovine hormone, however, differs significantly with respect to amino acid composition, molecular weight, and the number of peptide chains[2]. Since it appears desirable to extend the comparison, we wish to report on some aspects of the gross molecular configuration of the three proteins, based upon their electrostatic[3] and hydrodynamic[4] properties.

Since complete titration curves could not be secured, owing to the limited solubility of the bovine material and to the scarcity of primate preparations, the electrostatic parameters were estimated from a study of the ionization properties of tyrosine residues only. Absorption spectra of the growth-hormone solutions in 0.1 *N* KCl were recorded at 25° in the range of 280–300 mμ, the pH being varied between 9 and 13.5; allowance was made for background absorbancy, extrapolated from the region of 330–370 mμ as discussed by BEAVEN AND HOLIDAY[5]. The tyrosine content of each hormone was recalculated from these measurements (Table I). Spectrophotometric values were always greater than the earlier values obtained by means of the paper-dinitrophenylation method[1,6]; the former values appear to be the more reliable, however, since the latter procedure has well known limitations with respect to the analysis of tyrosine. Spectral changes associated with variation of the pH were reversible; they were also instantaneous, except at pH 13.5 where a slight increase in absorbancy was observed (less than 5%) during a period of 5 hours. The variation in molar

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