HYDROXYAPATITE AS A DEVELOPMENTAL FEATURE OF *SPIROSTOMUM AMBIGUUM*

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SUMMARY

1. Except in cells rapidly dividing in large vols. of a standard soil/wheat medium, X-ray diffraction diagrams of the ciliate *Spirostomum ambiguum* indicate the presence of hydroxyapatite at all ages and phases of the culture.

2. By centrifugation of crushed cells the fraction can be isolated that is responsible for most of the hydroxyapatite pattern. In the electron microscope, this fraction appears as dense round particles, up to 3 μ in diameter, with a periphery of needle-like projections in youthful cells, but containing many platelets in aged cells.

3. Electron micrographs of sections of animals containing these dense particles reveal a fine structure of radiating beaded threads of units approximately 215 Å $\times$ 65 Å, resembling similar structures observed in dentine. In time, these threads appear to become fused into plates.

4. The hypothesis is advanced that the presence of many organized ossicles of calcium phosphate is connected with a muscular burrowing phase, which is illustrated. The significance of the findings in relation to the evolution of the vertebrate skeleton is discussed.

INTRODUCTION

In a previous communication[1] the presence of bone salts was reported in the ciliate *Spirostomum ambiguum*, but no indication was given of any morphological feature responsible for the X-ray diffraction pattern, closely resembling that of hydroxyapatite, observed with preparations of whole animals.

Calcium deposits are common throughout the Protozoa, as exocellular calcified tests and shells and sometimes as discrete, often intricately figured, fabrications in the cytoplasm. In almost every case, however, the calcium salt is found as the carbonate, although deposits of other insoluble calcium salts have recently been described but not identified[2].

Large size and easy culture have made *Spirostomum* one of the best studied of the Ciliata, but no emphasis appears to have been placed on the presence of calcified objects within the cell. Thus BISHOP[3], in an earlier detailed account of cytoplasmic structures in *Spirostomum*, does not describe calcareous inclusions in the animal, although groups of dense particles are illustrated without comment in several of her drawings. More recently, RANDALL[4], in a series of studies on the fine structure of

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the contractile myonemes in *Spirostomum ambiguum*, mentions dense particles about 0.5 μ in diameter distributed throughout the cytoplasm.

In the Vertebrata, the secretion of calcium phosphate is not only vital for skeletal purposes, it also has a special metabolic significance in term of ionic homeostasis[5]. In this dual function as a labile source of Ca^{++} and PO_4^{---} (and to a lesser extent Mg^{++}) ions, and as an easily-constructed frame upon which large numbers of coordinated cells can operate, the close periodic association of hydroxyapatite crystallites and collagen fibres represents apparently the most advanced form of supporting tissue. It is therefore relevant to the study of the evolution of the vertebrate skeleton to know whether the storage of calcium phosphate in so "simple" an animal as *Spirostomum ambiguum* is also conditioned in the way it is in higher animals.

EXPERIMENTAL

S. ambiguum, from the Culture Collection of Algae and Protozoa, Cambridge, was cultured in a pasteurized medium of soil and wheat grains in tapwater. This pabulum promoted the most vigorous growth, although alternative combinations containing barley kernels and distilled water were also effective.

Flasks of medium were prepared by covering 1 vol. of soil with 7–10 vols. of tapwater. The medium was pasteurized at 80° for 1 h, and wheat grains, allowed to stand for 24 h after a brief boiling, were then buried just below the surface of the soil (5 grains for each 200 ml of culture). After standing for 7 days, the medium was inoculated with about 50 animals from a vigorous sub-culture, and a trace of boiled yeast extract was added. The organisms thrived best in diffuse daylight at 20°. During growth, detritus was not removed from the cultures, but from time to time wheat grains that had floated to the surface were re-buried in the silt.

At appropriate intervals, organisms were harvested from cultures varying in vol. from 200 ml to 10 l. Animals representative of the stage of growth were collected by disturbing the surface of the silt and allowing the coarse rubbish to settle. The swimming, thread-like cells (which were large and easily visible to the eye) were then removed by pipette or siphon and transferred to a larger vol. of distilled water, from which they were again selected after periods of time up to 24 h.

After 2 or more changes of distilled water, about 100 clean animals were then centrifuged to a small bulk and dried down on a glass block treated with a water repellent. For fractionation of cell contents by centrifugation, a large number of washed animals (up to 50,000) were first crushed between glass slides or, more effectively, were disrupted simply by freezing the packed cells.

Preparations for X-ray diffraction analysis were mounted dry on thin collodion film supported across a slotted brass holder, and photographs were taken with Cu Kα radiation collimated by lead-glass capillary, in a flat-film camera with a specimen-to-film distance of 4 cm.

Whole animals were prepared for examination in the electron microscope by a brief fixation in osmic acid vapour, followed by dehydration of groups of about 50 animals in dry absolute alcohol for several days. The cells were then embedded in butyl methacrylate polymer, sectioned with a glass knife, and generally examined without shadowing.

At every stage, calcium was estimated by the method of VON KÓSSA[6] and phosphate by the procedure described by SERRA and by FEIGL[7].

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RESULTS

Youth

After a resting period of some 7–21 days following inoculation, the animals usually multiplied rapidly. Cells were frequently seen dividing and on some occasions the inoculum of large cells 3 mm in length was quickly replaced by many smaller cells, sometimes only a few 100 μ long. In the early phases of growth, the supernatant fluid was often filled with many animals, either swimming freely or hanging vertically in characteristic "curtains". Conjugation, in one instance of epidemic proportions, was occasionally observed in thriving cultures.

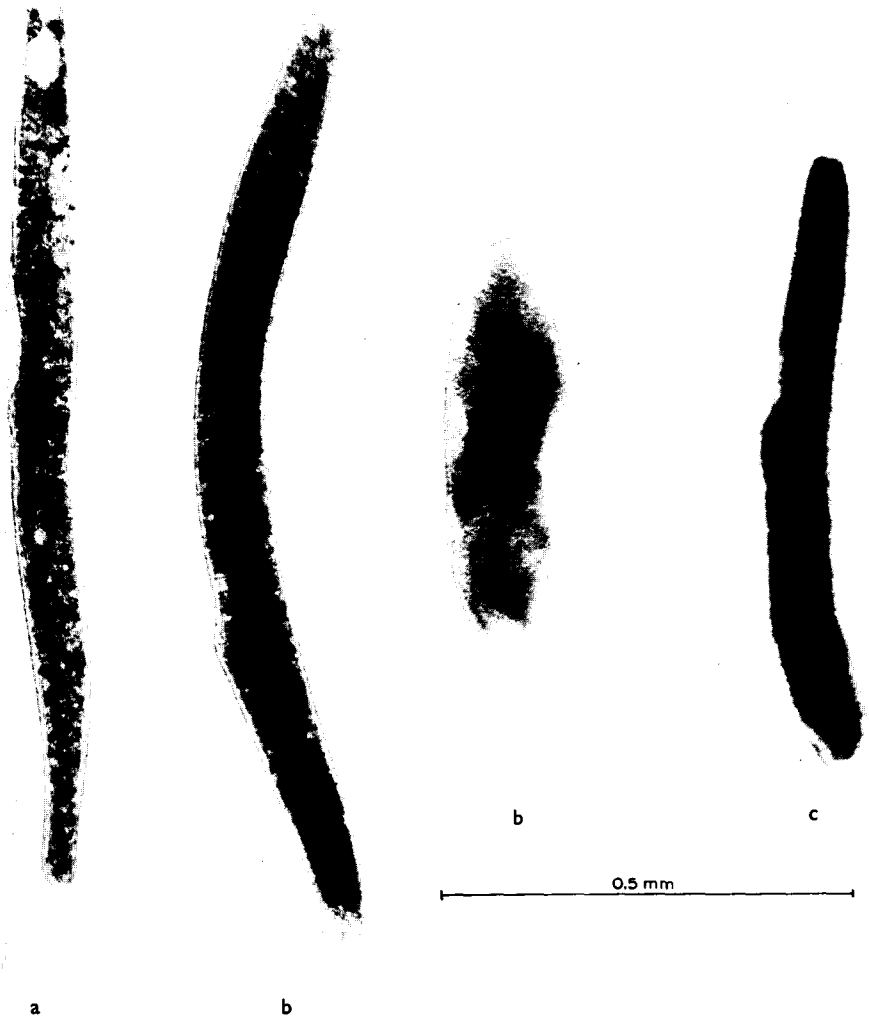

Fig. 1. *Spirostomum ambiguum* at various ages of the colony. (a) Young animal from a rapidly dividing colony, 3 weeks after inoculation. (b) Mature animals from a disturbed silt layer, 4 months after inoculation. (c) Old animal from below a silt layer, 1 year after inoculation.

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At this youthful stage, the organisms (a typical specimen is shown in Fig. 1a) were very active and the transparent cytoplasm stained negatively or feebly for calcium and phosphorus. In cultures of cells vigorously multiplying in larger (10 l) vols. of medium the X-ray diffraction pattern of washed and dried preparations of whole animals gave either vague, or no, indications of hydroxyapatite, although a number of other well defined continuous rings, as yet unidentified, were often present (Fig. 2a).

Maturity

After a few weeks, calcium phosphate was detected in increasing amounts in the cells, which were by now mostly of similar size, appeared optically denser than at the onset of growth, and were found predominantly among the rubbish on the

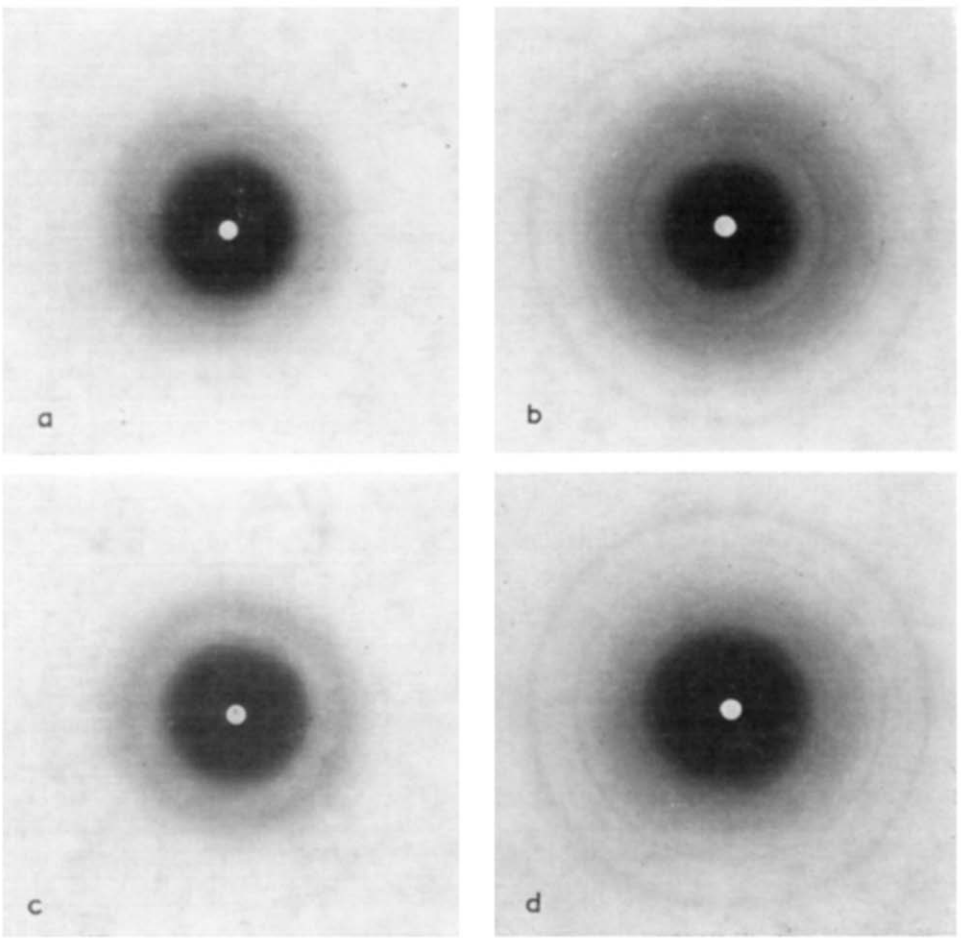

Fig. 2. X-ray diffraction patterns from random samples of 100 cells of *S. ambiguum* mounted on collodion. (a) Vigorously thriving colony in 10 l of culture fluid, 5 weeks after inoculation. (b) Mature cells, gathered from supernatant fluid, 4 months after inoculation. (c) Early maturity, several weeks after inoculation. (d) Aged colony below the silt, 1 year after inoculation.

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surface of the silt in the culture flasks. Two mature animals, one extended and one contracted into the characteristic spirally-distorted spindle, are shown in Fig. 1b.

The X-ray diffraction patterns of whole animals from mature colonies always indicated hydroxyapatite, often with additional rings like those found with rapidly multiplying animals (Fig. 2b) but sometimes, from animals at an earlier stage of growth, with more diffuse reflexions suggesting protein (Fig. 2c). There appeared to be little difference in the phosphate accumulation in cells grown with an exposed or buried food supply, from various areas of the same culture, in light or darkness, or in various combinations of media.

Suspensions of débris from mature animals crushed in ice could be separated into fractions by centrifugation. The heavier and lighter fractions, when washed, dried and suitably mounted, gave vague X-ray diffraction patterns suggesting protein, but the intermediate fraction, separated at 4000 $\times$ g for 10 min gave, when dried and mounted, the major bulk of the X-ray diffraction diagram of hydroxyapatite. In the electron microscope, this fraction was seen to consist of large numbers of dense round bodies, varying from 0.5–3 μ in diameter, often clustered together and surrounded by the remnants of a membrane. In carefully focussed transmission electron micrographs of these clusters, illustrated in Fig. 3, for example, the perimeter of each particle appeared as the termination of many needle-like projections.

Sections of similar mature animals revealed many groups of dense round bodies corresponding in size and peripheral structure (if allowance is made for compression by the knife) to the centrifugally-separated particles giving the bone-salt pattern. The groups of particles, like those shown in Fig. 4, were scattered throughout the cytoplasm and each group was usually contained within a well defined boundary. By examining numbers of sections of particles in various groups, the fine structure of each particle in mature *S. ambiguum* could be represented by a radially-disposed network of threads, disordered in the centre, but diverging more regularly towards the perimeter.

At higher powers, as in the electron micrographs in Figs. 5a and 5b, the dense threads were resolved into beaded structures of a thickness (about 65 Å) corresponding to the peripheral needles of the centrifugally-separated hydroxyapatite particles. The beads were roughly cigar-shaped, and from an average of some 200 observations the mean size of each bead was about 215 Å long by 65 Å at the thickest part. As many as 5 of these cigar-shaped units were counted end-to-end in one needle, but more usually, 2 or 3 represented the most that could be seen clearly in any one region of the thread. There was a close resemblance, both in shape and size, between the units strung together in the needles of the mature *Spirostomum* particles and the recently observed fine detail in dentine sectioned with a glass knife[8]. In Fig. 5c the arrangement of beaded threads in dentine can be compared with the similarly prepared *Spirostomum* structure in Fig. 5b.

Age

In ageing cultures, usually about 5 months after inoculation, the cells became progressively smaller and more optically dense (Fig. 1c). In old cultures, upwards of a year following inoculation, the animals became so calcified that movement was grossly restricted. In some cases the organism, though capable of swimming, could not flex its axis by more than a few degrees, in contrast to young or mature animals,

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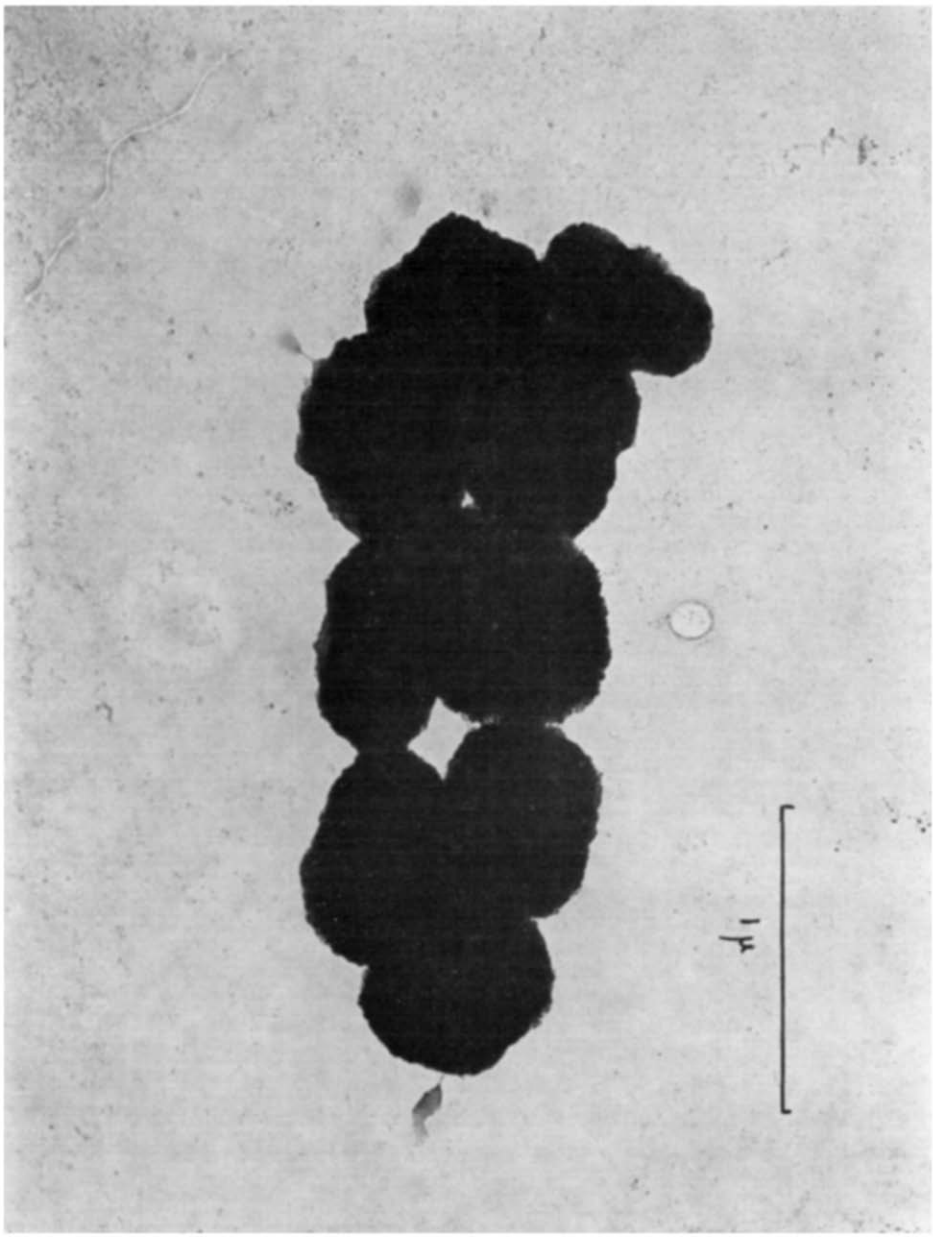

Fig. 3. A cluster of particles from the 4000 × *g* × 10 min fraction of crushed *S. ambiguum* cells. Random sample of a colony 2 months after inoculation. Unshadowed.

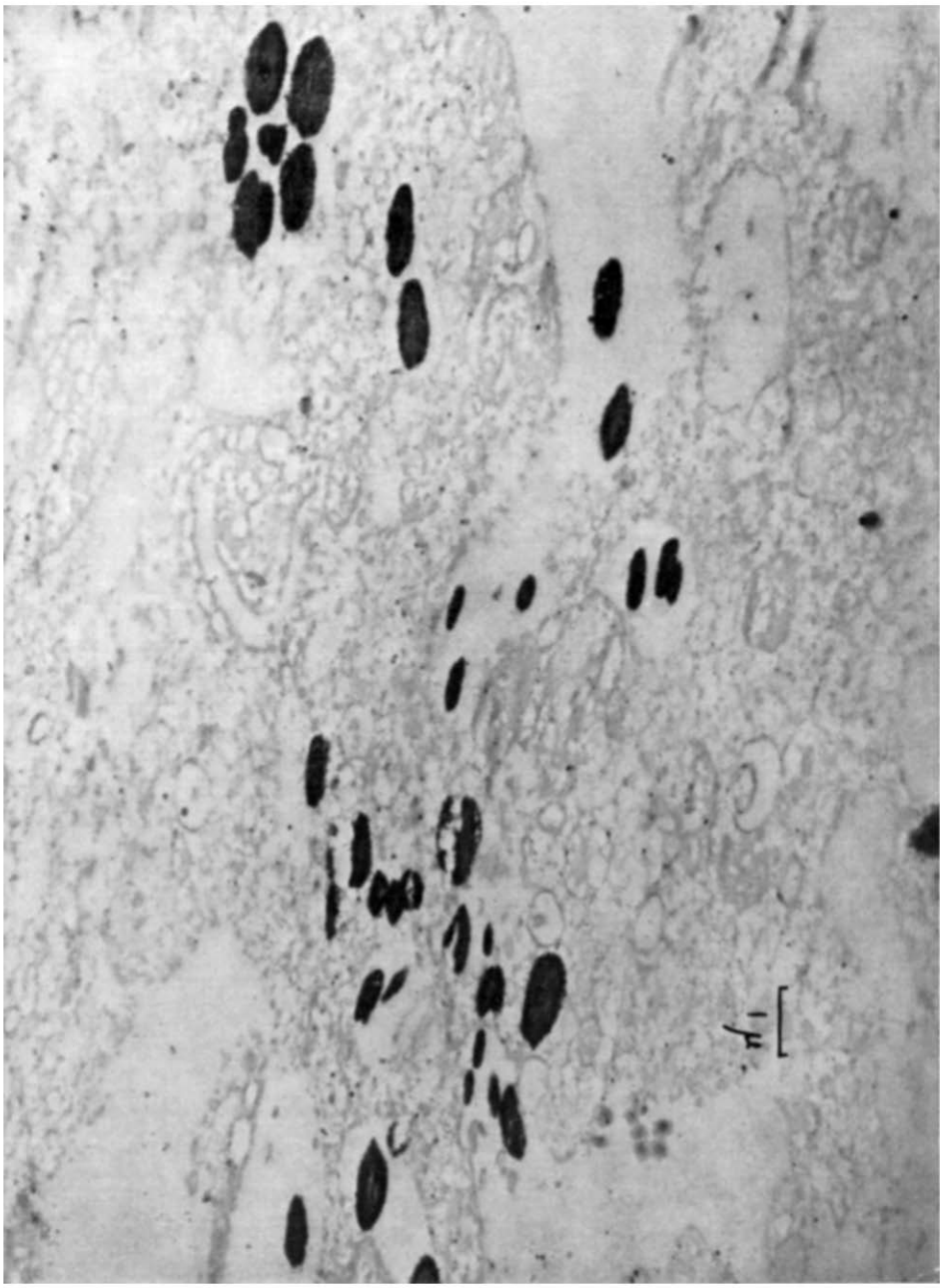

Fig. 4. Section of *S. ambiguum* from the same source as Fig. 3. General view showing groups of particles. Lightly fixed in osmic acid. Unshadowed.

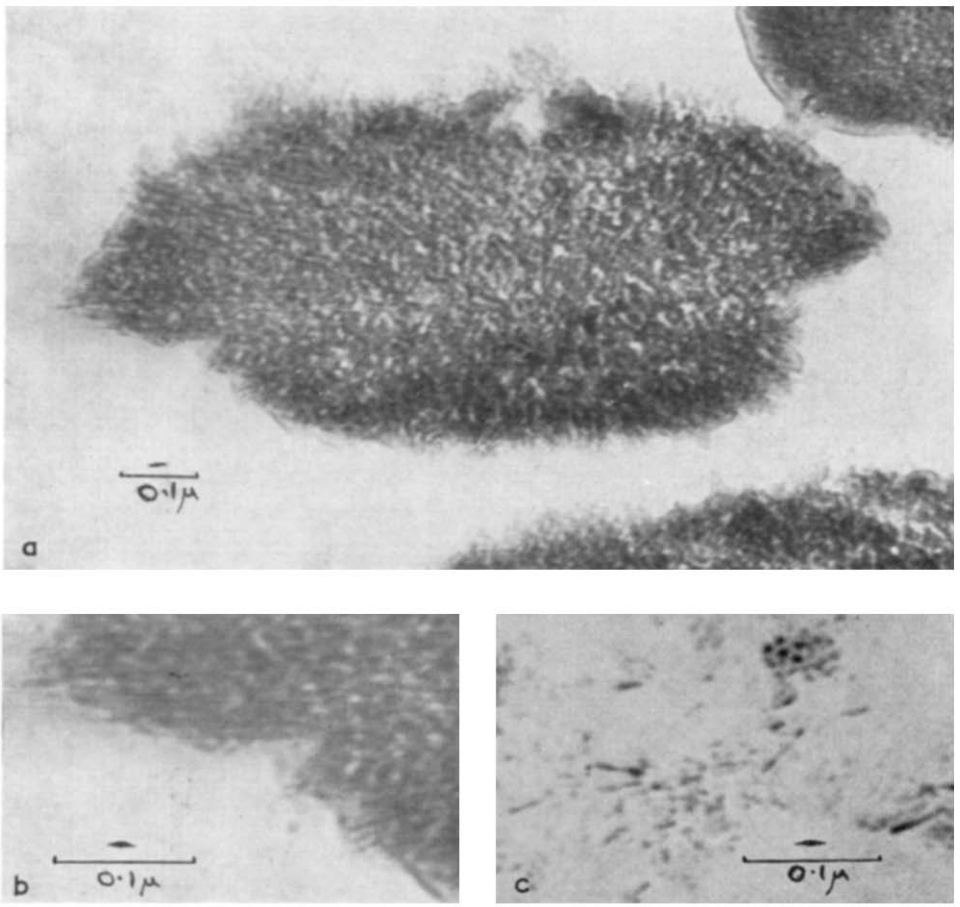

Fig. 5. Fine structure of ossicles from mature *S. ambiguum* compared with dentine. All sections are unshadowed. A silhouette of a single average bead of 215 Å × 65 Å is shown for reference. (a) Enlarged micrograph of an ossicle from the preparation shown in Fig. 4. (b) Detail of the edge of (a) above. (c) Detail of a section of dentine from human immature 1st molar tooth. Random orientation of powdered fragment.

which frequently avoided obstacles by bending through 180°. The dense and rigid cells stained strongly for calcium and phosphorus, and the X-ray diffraction pattern of whole animals was characterized by strong reflexions of hydroxyapatite with no suggestion of protein (Fig. 2d).

As in the case of mature animals, the intermediate centrifuged fractions of crushed cell debris gave the major bulk of the hydroxyapatite X-ray diffraction pattern. In the electron microscope, however, the particles (illustrated in Fig. 6) associated with this pattern were no longer composed only of beaded thread-like networks but also appeared as aggregation of plates, often of large size, but all contained within, or confined by, vesicles similar in size to the ossicles observed in mature animals. The X-ray diffraction pattern of these particles from old cells suggested, however, that the crystallite size was much smaller than the "crystals" (often as much as 1 μ long) observed in the electron microscope. The compound character of

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Fig. 6. Particles from a 4000 × *g* × 10 min fraction of *S. ambiguum*. Random sample of an old colony. Gold-palladium shadowed.

these plate-like deposits was confirmed by intermediate stages seen in animals from older cultures, where there appeared to be a progressive coalescence of the beaded threads into aggregates, often fused together into denser areas but still discernable as arrays of smaller particles.

Burrowing phase

A universal feature of all cultures was the commencement of a burrowing habit a few weeks after inoculation and usually coincident with the depletion of food supplies in the supernatant liquor. The mining of the silt, which appeared to take place even when the food was not buried, was preceded by the congregation of animals into one area. At first, the burrowing activity was restricted to communal entry between spaces in the upper layers of silt, shown in Fig. 7, but at a later stage tunnels and galleries were driven into the lower strata of firmer soil (Fig. 7 inset).

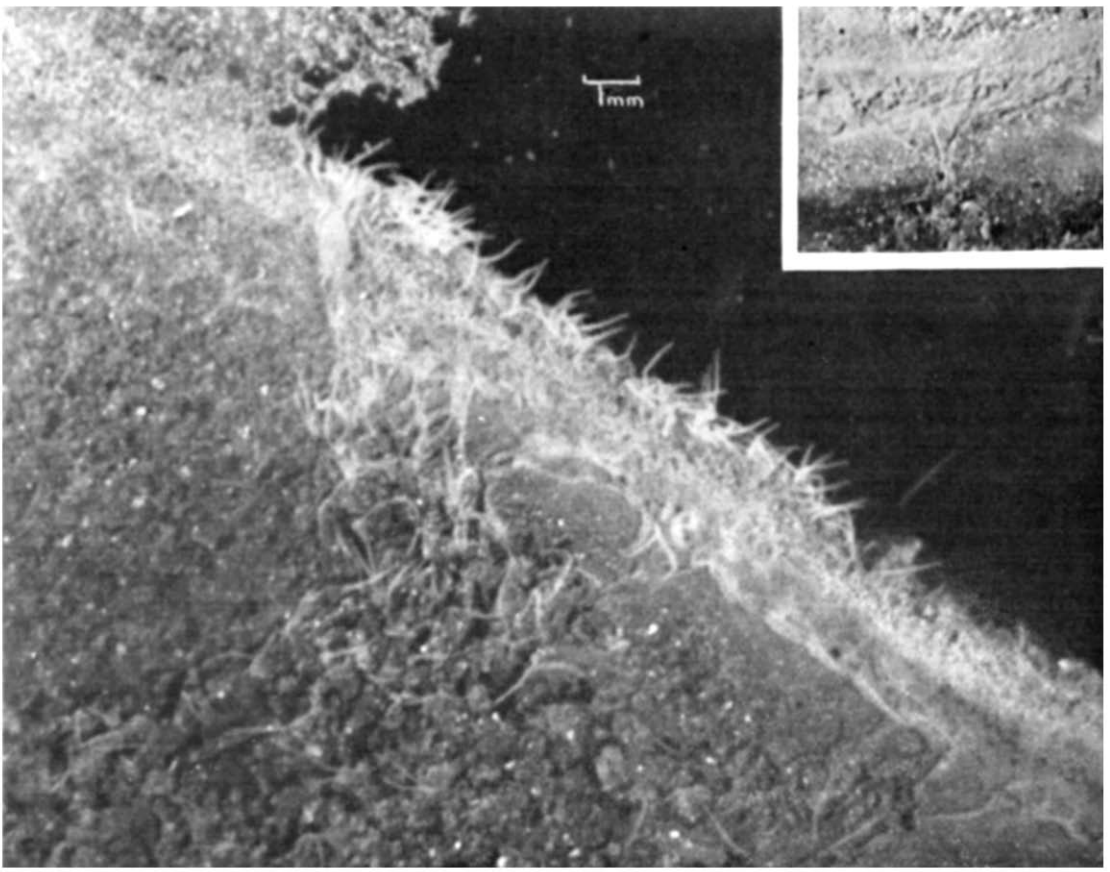

Fig. 7. Macrograph of collective entry of *S. ambiguum* into sub-silt layers. Groups of animals can be seen congregating at the surface on the left. Regular pathways can be discerned between silt particles. Photographed through the side of a 1-l Roux Bottle containing a 5-month-old culture. (Inset: Detail of an area slightly to the right of the general picture shown. A small network of galleries can be seen.)

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The tunnels were made by intermittent contraction of the spiral myonemes, after insertion of either posterior or anterior end of the animal into a crevice at the working face of the tunnel. The increase in diameter accompanying the corkscrew motion of the cell (the relative difference before and after contraction can be seen in Fig. 1b) served to prise apart a gallery of suitable width. In older cultures the collective activity of many animals in one area resulted in small mounds, like miniature molehills. These mined areas in the silt provided a rich source of material, and on some occasions dredging with a pipette excavated animals from depths of several centimetres.

Decalcification

Decalcification of ossified cells in neutral 2–10 % ethylenediamine tetra-acetic acid (EDTA) resulted in the disappearance of the X-ray diffraction pattern of hydroxyapatite in every case without any definite indications of residual reflexions that would suggest collagen. In the electron microscope, the dense particles became very diffuse after decalcification and no fine structure could be seen. It was found that present techniques for the removal of calcium, particularly in thin sections, were unable to prevent complete disintegration of the calcified structures, the quantitative examination of which must await the culture of very large numbers of cells.

Except in animals freshly inoculated into large volumes of culture medium, calcium phosphate did not disappear from the organisms observed. Starvation produced a diminution in size and activity but the hydroxyapatite pattern remained. Sudden changes of culture conditions (sunlight, darkness and temperature) resulted in no gross differences in the calcium phosphate present.

Summary of changes

Fig. 8 represents diagrammatically the general development of the ossicles in *S. ambiguum*, provided, that is, that the animals are allowed to mature in an unchanged culture medium. There is no evidence that during the growth of new cells the ossicles from the parent inoculum revert to the youthful phase, or that during rapid growth, when the calcium phosphate disappears from the cells, the aged crystalline ossicles are not discarded in favour of fresh deposits. If the old, highly calcified, cells can mobilize their phosphate reserves, there is no indication of the way in which this is accomplished. The overall picture seems to be that of a permanent retention of organized calcified particles which gradually become denser, fused, and more numerous with time. The factors involved and the stimuli that induce such morphological changes must await further experiment.

DISCUSSION

When considering the morphological changes that take place with time in Protozoa there is always considerable difficulty in relating the age of the colony to the age of individual cells. Sudden access to food might create pockets of dividing cells; removal of stimuli might result in some rapid change in cytoplasmic contents. If, however, a colony can be said to "run down" after passing the steady state following the growth peak, then there is some validity in the suggestion put forward by MUGGLETON AND DANIELLI[9] that under conditions of equilibrium with the food supply

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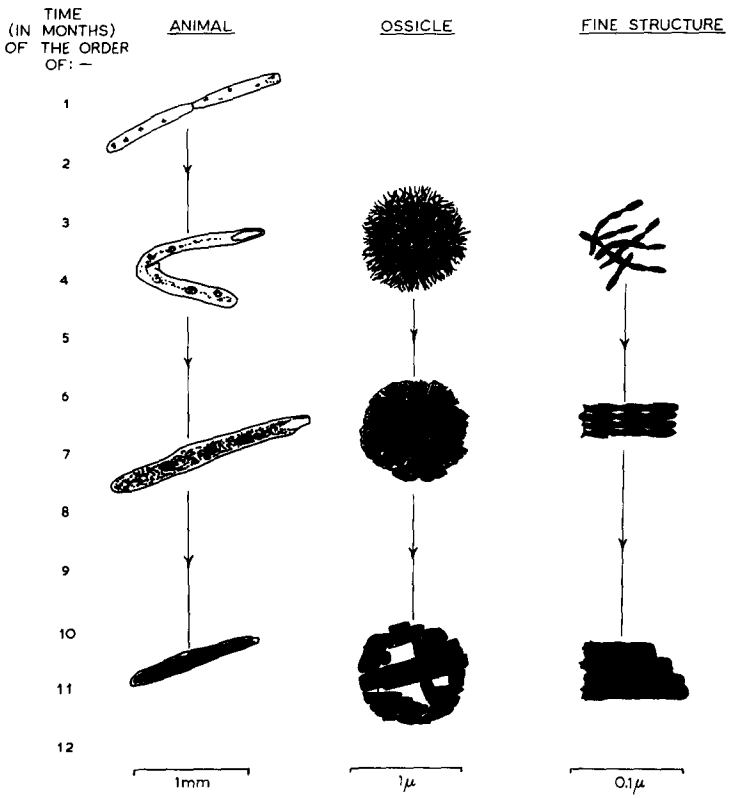

Fig. 8. Development of ossicles in *Spirostomum ambiguum*.

there is a definite life-span in the protozoan cell. In the present experiments on *S. ambiguum* a complete study of the life of a single organism is not practicable and the changes observed are those experienced by the colony collectively. Positive changes in random samples must nevertheless reflect similar changes in individual animals, and the steady progress of the sum total towards old age might very well signify the mortal character of the individual cell.

The storage of calcium phosphate within the cytoplasm seems to be a characteristic feature of *Spirostomum ambiguum*, and the development of dentine-like particles and the changes that take place with time strongly parallel the course of events in the vertebrate skeleton. The relationship of crystallite to organic matrix in bone has been demonstrated both by X-ray[10] and by electron-microscope[8,11] studies. In the protozoan "dentine" the storage of phosphate appears to follow the now familiar pattern of deposition of particles on and/or within some fibrillar nucleus until chains of units are formed. The cigar-shaped unit of about 215 Å × 65 Å would appear to be the fundamental crystallite, and this resemblance to the generally accepted size for the primary particles in metazoan bone, with its supposed association with 1/3 the collagen macro-period (640 Å), is significant. It is likely that there is a common "calcification plan" throughout nature and that, in the case of phosphate, the varicose, possibly circumfibrillar, deposition of mineral salts on collagen, or some

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precursor of collagen, is the universal means of secreting hydroxyapatite. In *Spirostomum* and probably in metazoan bone[11], the welding together of these strings of microcalculi results in time in plates or sheets, but in the protozoan sudden rapid growth might result in complete mobilization of the stored phosphate. This latter possibility suggests that some emphasis should be placed on the importance of these structures in coping with sudden metabolic changes, and for this function the youthful system of separated beaded threads enmeshed in a labyrinth offers a large surface area for ionic exchange while preserving some structural strength. With alignment of the increasingly calcified parallel arrays of threads, there will be an increase of mechanical strength along preferred axes, as is the case in metazoan bone, at the expense of exchange surface area. With fusion of the parallel arrays into sheets the available exchange surface will be still further reduced and the crystalline embrittlement of the structure will proceed to a point where the system possesses neither accessibility or strength.

Evolutionary significance

LULL[12] stressed the relationship between the vertebrate skeleton and the dynamic way of life. In predators, muscularity may demand more from the precipitated salt than support for cells engaged in intense metabolic activity, and in this way the skeleton might be regarded as a lending and borrowing phosphate-bank as well as a convenient source of Ca and Mg ions.

Spirostomum ambiguum may thus represent a unicellular arrangement of stored phosphate readily available for the phosphorylative steps necessary for muscular energy during the tunnelling phase and for the creation of new muscle tissue and progeny when further food, particularly protein, has been won. The cycle of starvation, hard work and gluttony that occurs during the life of this protozoan follows a pattern similar in outline to the intermittent feeding habits of the Vertebrata. The very muscular character of an animal otherwise excellently equipped with cilia and capable of rapid and controlled swimming must be explained in terms of some other phase of life demanding a more intense expenditure of energy, and to this end the burrowing habit may be a means of survival in the face of a shrinking supply of the more easily accessible food. The worm-like form of *Spirostomum*, allied to a muscular ram, is admirably adaptable to a tunnelling existence, and the stresses applied during the mining operations would demand an articulatable skeleton. The frequent changes of activity involved could very well be responsible, through attendant alterations in mechanical and metabolic pressure, for changes observed in the fine structure of the ossicles.

Thus it would appear that the price of a full and active life in *Spirostomum ambiguum* is the osteo-arthritis of old age, and it may be that in the Vertebrata, too, increasing calcification with time is a sign of anxious expectation for a metabolic event that never occurs.

ACKNOWLEDGEMENTS

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THE SIZE AND SHAPE OF THE APATITE CRYSTALLITES IN BONE AS DETERMINED FROM LINE-BROADENING MEASUREMENTS ON ORIENTED SPECIMENS

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SUMMARY

Line-broadening measurements on X-ray diffraction patterns from oriented specimens of bone with a well-ordered internal structure have shown that the line-broadening effects observed are caused both by a limitation in size of the apatite crystallites and by lattice distortions. The results indicate that the apatite crystallites in bone form long rods which are probably deformed by mechanical forces. The rods have an average diameter of 40–45 Å and their average length is in the order 600–700 Å. These findings are regarded as being much more reliable than those previously deduced from X-ray data.

INTRODUCTION

Several trials have been made earlier in order to deduce the crystallite size of the bone salt from the broadening of the Debye–Scherrer lines in X-ray diffraction patterns of powdered specimens. A first rough estimation by DE JONG[1] in 1926 showed that the crystallites were very small, probably containing not more than a few hundred apatite molecules. BALE, HODGE AND WARREN[2] found in dentin an average size of 240 Å,

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