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# CALCIUM PHOSPHATE GRANULES IN THE HEPATOPANCREAS OF THE BLUE CRAB *CALLINECTES SAPIDUS*

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ABSTRACT

The hepatopancreas of the adult male blue crab *Callinectes sapidus* in intermolt was found to contain substantial amounts of calcium, magnesium, and inorganic phosphorus, averaging about 260, 20, and 250 μg-atoms per g wet tissue, respectively, accounting for over 10% of the tissue dry weight. Electron microscopy of the intact tissue showed three qualitatively different granular structures having electron densities suggestive of high mineral content. After fractionation of the tissue using centrifugal techniques, almost 95% of the total mineral was found to reside in a heavy, nonmitochondrial particulate fraction(s). The bulk of the low-speed pellet consisted of relatively dense, roughly spherical granules 1–5 μm in diameter, which could be considerably purified by repeated suspension in water and low-speed sedimentation. In the electron microscope the isolated granules appeared basically similar to one of the three characteristic types of electron-dense granules seen in the intact tissue. Although the freshly isolated granules lost approximately 50% of their wet weight when dried at 105°C, only 10% more was lost upon dry ashing at 450°C, suggesting a fairly low content of organic material. Chemical analysis revealed calcium, magnesium, and inorganic phosphate at 5.7, 2.1, and 4.4 μg-atoms per mg dried granules, respectively, accounting for 69% of the dry weight of the fraction. By specific enzymatic assays, the freshly isolated granules were found to contain ATP, ADP, and AMP at levels of 0.13, 0.03, and 0.01 μmol/mg, or 8% of their total dry weight. The remainder of the total phosphorus contributed an additional 3%, whereas carbonate, citrate, oxalate, and protein each constituted no more than 1%.

The mineral granules of the crab hepatopancreas appear to function as storage forms of calcium and phosphate during the intermolt period. This tissue appears promising as a model for study of the cellular events associated with biological calcification, since conventional biochemical techniques can be employed. Furthermore, the major mineralized component of the tissue can be obtained in large amounts for direct study by a simple fractionation procedure.

In preceding papers it was shown that mitochondria isolated from the hepatopancreas of the blue crab *Callinectes sapidus* are capable of vigorous respiration and phosphorylation (1) and are also capable of energy-dependent accumulation of large amounts of $Ca^{2+}$ and phosphate from the suspending medium (2). After such accumulation of $Ca^{2+}$, large electron-dense deposits or clusters

of needle-like crystals of calcium phosphate could be visualized in the matrix of these mitochondria by electron microscopy. The capacity of the crab hepatopancreas mitochondria to transport $Ca^{2+}$ is of special interest because this decapod crustacean appears to be among those species which, before molting, resorb $Ca^{2+}$ from the mature exoskeleton and store much of it in soft tissues of the gastrointestinal tract; after molting occurs this $Ca^{2+}$ is rapidly mobilized for calcification of the new shell. The storage of very large amounts of insoluble mineral in the hepatopancreas of this animal appears to be a potentially useful model for experimental testing of a hypothesis (3) concerning the possible role of mitochondria in biological calcification.

In this paper we report the occurrence, size, composition, and ultrastructure of $Ca^{2+}$-containing granules in the cytoplasm and extracellular space of the hepatopancreas of *C. sapidus*. These granules are considered in relation to the $Ca^{2+}$-transporting activity of the mitochondria in this tissue and in relation to the possible role of mitochondria in the biological deposition of solid calcium phosphate in calcification processes. Relationships to the formation of calcareous corpuscles or granules in cestodes and other invertebrates are also discussed.

## METHODS

Animal selection, tissue removal, homogenization, and fractionation through the low-speed centrifugation step were carried out essentially as described in a preceding paper (1), except that the homogenization medium contained no bovine serum albumin (BSA) or EDTA. For chemical analysis whole tissue and its soluble fraction were obtained by homogenizing undiluted tissue, then centrifuging it at 140,000 *g* for 30 min. Analyses for Ca, Mg, and phosphate were done as before (2). Total phosphorus was determined after wet ashing with sulfuric acid. To determine carbonate samples were acidified, the liberated $CO_2$ was absorbed in alkali, and the resulting carbonate was precipitated with $Ba^{2+}$ and weighed as barium carbonate. Protein and ninhydrin-positive material were determined as before (2). Dry weights were obtained on samples heated at 105°C for 18 h and cooled in a desiccator over silica gel. Dry ashing was carried out in a muffle furnance by gradually increasing the temperature until charring occurred, heating at 450°C for another 72 h, then cooling, and weighing. ATP, ADP, and AMP were determined by the specific enzymatic methods used before (1), after solubilizing the granules in an excess of 0.1 M EGTA, pH 7.5.

Electron microscopy of the intact hepatopancreas tissue and subfractions was carried out essentially as described before (2) except in the case of the isolated granules. Because of the higher mineral content in this fraction, infiltration was exceedingly difficult with our routine procedures. Thus, the low viscosity embedding medium of Spurr (4) was used with infiltration taking place in increasing ratios of plastic to ethanol over a total of 8 days.

## RESULTS

### *Mineral Granule Fraction Obtained by Fractionation of Hepatopancreas Homogenates*

During the course of isolation of the mitochondrial fraction of crab hepatopancreas by differential centrifugation, as described in a preceding paper (1), it was found that the first (low speed) centrifugation step employed to sediment the nuclear fraction invariably yielded a pellet of very large size and unusual appearance. The fastest sedimenting and by far the bulkiest portion of this fraction was a dense, white, chalky sludge, the characteristics of which suggested a high mineral content. Resuspension of this portion of the pellet and examination by light microscopy showed it to consist almost entirely of refractile, roughly spherical bodies 1–5 μm in diameter. These bodies stained intensely with Alizarin Red S, a fact which suggested that they contained calcium (5). This dense material in the first low-speed pellet was then "purified" by skimming off and discarding the less dense portions of the pellet. Repeated suspension in cold water and low-speed sedimentation of the chalky residue yielded a white, gritty, grossly homogeneous pellet, hereafter called the *mineral granule fraction*, which dried to a white powder *in vacuo*. Although the yield of this material was about 30 mg/g whole wet tissue, the actual amount of the mineral granule material in the original homogenate was probably two- to threefold higher: considerable losses were incurred in order to achieve a minimum of contamination with other cell fractions.

### *Mineral Composition of Crab Hepatopancreas Homogenates*

The mitochondrial fraction of crab hepatopancreas has already been found to contain large amounts of calcium and phosphate (2). To obtain a more complete picture of the mineral distribution within this tissue, the total calcium, magnesium, and phosphate were determined for three different animals on the whole tissue and its high-

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TABLE I

*Mineral Ion Content of Hepatopancreas of Blue Crab*

| Experiment | Fraction | Mineral content (μg-atoms/g wet tissue) | | | |
|---|---|---|---|---|---|
| | | $Ca^{2+}$ | $Mg^{2+}$ | $P_i$ | P total |
| 1 | Whole tissue | 269 ± 4.9 (3) | 27.0 ± 1.1 (3) | 248 | 300 |
| | Supernate | 5.9 ± 0.02 (3) | 5.4 ± 0.07 (3) | 14.3 | 35.9 |
| 2 | Whole tissue | 226 ± 8.0 (3) | 15.4 ± 0.3 (4) | | |
| | Supernate | 11.1 ± 0.4 (2) | 3.1 ± 0.1 (2) | | |
| 3 | Whole tissue | 282 ± 7.8 (4) | 18.3 ± 0.5 (4) | | |
| | Supernate | 8.5 ± 0.2 (2) | 4.5 ± 0.1 (2) | | |
| | Whole rat liver | 0.44* | 6.7* | | |

Figures are given as the average ± SD of the number of determinations in parentheses.
* Recalculated from the data of Thiers and Vallee. 1957. *J. Biol. Chem.* **226**:911.

speed soluble fraction, obtained as described under Methods. It is seen (Table I) that this tissue contains exceedingly large amounts of these mineral ions. For example, the $Ca^{2+}$ content (~260 μg-atoms per g wet tissue) is 300- to 400-fold greater than the values reported for normal rat liver. The extraordinary magnitude of the mineral content for what is nominally a parenchymal tissue can be better appreciated by the fact that the sum of the calcium, magnesium, and inorganic phosphate accounts for over 10% of the dry weight of the hepatopancreas.

The data in Table I also show that only about 5% of the $Ca^{2+}$ and $P_i$ remains in the soluble fraction of the tissue after centrifugation at 140,000 *g* for 30 min. Although the mitochondrial fraction has already been found to contain as much as 1,000 ng-atoms $Ca^{2+}$ per mg protein (2), this amount corresponds to only 2 μg-atoms of $Ca^{2+}$ per g whole tissue, or only about 1% of the tissue's total $Ca^{2+}$. It was therefore concluded that some sedimentable fraction other than the mitochondria must contain nearly 94% of the $Ca^{2+}$ of the whole tissue. Most of this remainder is apparently localized in the mineral granule fraction described above.

## *Analysis of Mineral Granule Fraction*

Analyses carried out on aliquots of pooled, washed mineral granule preparations are reported in Table II. Because of the obvious losses of granule material during the washing and sedimentation procedures, the analytical data are related to a final weight of dry granules rather than to an initial weight of whole tissue from which the granules were isolated. The data show that calcium, magnesium, and inorganic phosphate make up almost 70% of the dry weight of the granule fraction. The granule fraction thus represents a rather heavily mineralized tissue component; for comparison, the mineral content of the extracellular ("matrix") fraction of adult mammalian bone is somewhat less, about 65%. Specific analysis for carbonate in the granule fraction showed that it made up no more than 1% of the total granule weight. Thus phosphate is the predominant mineral anion in the hepatopancreas granule fraction, despite the fact that the mineral portion of the crab exoskeleton is thought to be largely calcium carbonate. This disparity has also been documented for the closely related species *Carcinus maenas* (6). No other inorganic components

TABLE II

*Chemical Composition of Granule Fraction Isolated from Hepatopancreas of Blue Crab*

| | μmol/mg granules* | Weight percent* | |
|---|---|---|---|
| Ca | 5.7 | 22 | |
| Mg | 2.1 | 5 | |
| $P_i$ | 4.4 | 42 | |
| ATP | 0.13 | 7 | 80 |
| ADP | 0.03 | 1 | |
| AMP | 0.01 | (<1) | |
| Other P | 0.3 | 3 (as $P_i$) | |
| $CO_2$ | | <1 | |
| Citrate | | <1 | |
| Oxalate | | <1 | |
| Free amino acid | | <2 | |
| Protein | | <1 | |

* Based on weight obtained after heating at 105°C and cooling over silica gel.

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appeared to be present in significant amounts, since the sum of calcium, magnesium, and total phosphorus (as phosphate) amounted to 75% of the granule dry weight and since the isolated granules lost a further 20% of their dry weight upon ashing at 450°C. The granules could be completely dissolved in 1–2 N HCl to yield a clear, colorless solution. Neutralization of this solution with NaOH caused reprecipitation, presumably of insoluble calcium and magnesium salts.

The organic component(s) of the granules were then examined. Analyses for protein and ninhydrin-positive material in 1 N HCl solutions of the granule fraction revealed no more than 1–2% by weight of amino acids, peptides, or protein. Moreover, the essentially complete solubility of the granules in 1 N HCl and in neutral EGTA solutions suggested that lipid was not a quantitatively important constituent of this fraction. Thus the granules did not appear to contain significant amounts of the organic matrix components most commonly associated with mineral deposits in other kinds of tissue, namely proteins, glycoproteins, and phospholipids. However, the presence of a significant amount of organic material in the granule fraction was suggested by conspicuous charring and a 20% weight loss of the granules drying dry ashing. Moreover, considerable phosphorus (~0.6 μmol/mg of dry granules), undetectable as orthophosphate, was released in inorganic form by wet ashing and was thus present in covalent linkage, possibly to some organic moiety. The UV spectrum of a 2 N HCl solution of the granules showed the presence of absorption maxima at 209 and 258 nm, with minimum absorption at 230 nm. This UV-absorbing component appeared to coprecipitate and redissolve with the insoluble mineral of the granules. These data suggested the presence of nucleotides in the granules. Based on the known extinction coefficient for ATP, the 258 nm absorbing material amounted to 0.19 μmol ATP per mg dry weight, or about 9.5% of the dry weight of the granules. It is noteworthy that 0.6 μmol of inorganic phosphate per mg dry weight appeared on wet ashing, approximately the amount expected if the UV-absorbing material were ATP. By specific enzymatic assay of the EGTA extracts, it was found that the granules contained 0.13 μmol ATP, 0.03 μmol ADP, and 0.01 μmol AMP, for a total of 0.17 μmol adenine nucleotide per mg dry granules, in good agreement with the spectrophotometric determination.

The mineral granules contained no uric acid, cysteine, citrate, or oxalate, as determined by qualitative tests which would have detected these substances at levels of 1% by weight.

### *Ultrastructural Studies*

An electron microscope survey of thin sections of intact hepatopancreas tissue was carried out to determine the content and distribution of solid-phase calcium and phosphate in the intact tissue, as indicated by the presence of electron-dense deposits. The usual cytological features of epithelial cells, including nucleus, mitochondria, and a well-developed brush border were observed. Distinctively, as shown in Fig. 1, these cells contain large numbers of cytoplasmic vesicles averaging about 0.6 μm in diameter interspersed among the mitochondria; a prominent zone (about 1 μm wide) near the epithelial surface is largely devoid of these vesicles. Smaller numbers of larger vesicles are also seen. These vesicles typically contain little dense-staining material. The intercellular contacts are characterized by extensive dense-staining regions which are probably the sites of complex gap junctions.

In addition, several unusual structures having distinctive ultrastructural characteristics are observed. Of these, the most frequently seen is an extremely large granule measuring as much as 20 μm in diameter and having staining properties similar to lipid (triglyceride) droplets; they are designated type I granules. As reported earlier (1), crab hepatopancreas tissue is exceedingly rich in low-density lipids, which must be skimmed from whole tissue homogenates before mitochondria can be obtained by centrifugation. However, these huge structures, unlike typical lipid droplets, often exhibit a pronounced layered structure, especially at their peripheries. Also, the presence of nonstaining lines or fractures within these structures, as well as the behavior during sectioning of embedded tissue containing many of these structures, suggests that they contain some hard (mineral?) material which is infiltrated with difficulty (see Fig. 1). Furthermore, as can be seen in Fig. 2, discrete regions of variable staining properties are often apparent within these large structures. Thus, it would appear that these structures are sometimes heterogeneous in composition. No distinct membrane has been seen surrounding these very large structures even when observed in the electron microscope at high magnifications.

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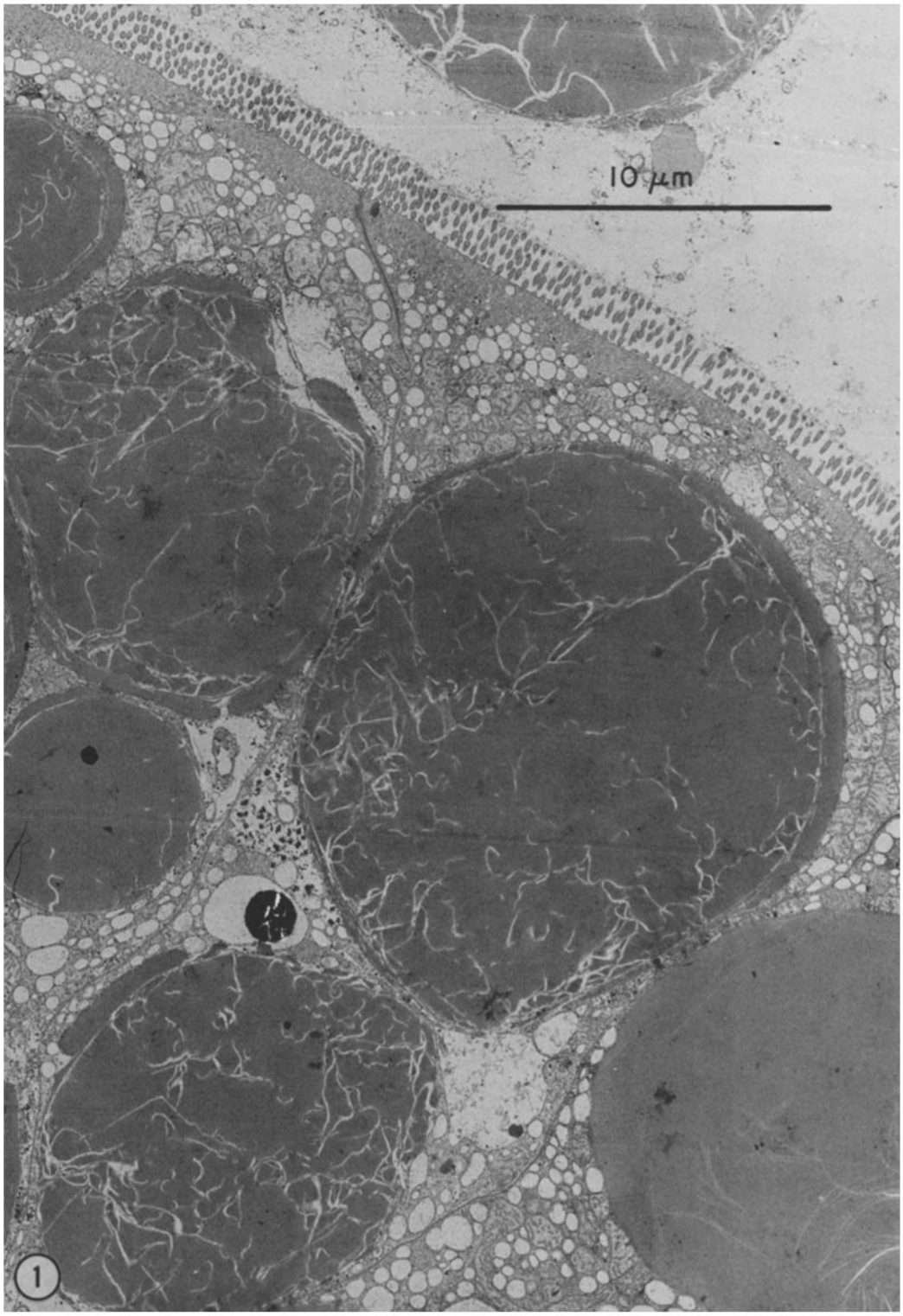


FIGURE 1 Thin section of crab hepatopancreas showing huge type I granules of intermediate electron density. Nonstaining lines or fractures can be seen. Peripheral layer around the large granules is apparent. Fixed with glutaraldehyde and osmium. Sections stained with uranyl acetate and lead citrate. × 5,250.

A second ultrastructurally distinctive feature consists of extremely dense-staining spherical structures measuring about 3.0 μm in diameter (type II granules). They appear to become condensed, to retract, or to become peripherally solubilized during tissue preparation, as indicated by the wide, nonstaining region which surrounds them. The electron-dense cores appear to be hard, mineral structures, since most have evidently undergone fracture and are frequently displaced during sectioning. Clusters of dense-staining, needle-like crystals can be seen around the periphery of these spherical bodies. Their appearance suggests that in vivo this dense material may be compartmented within a large membrane-enclose vesicle, which conceivably could be formed by coalescence of the smaller vesicles (see Fig. 3). Type I and type II structures are found frequently within the same cells.

The third type of distinctive structure (type III granules) has an especially striking appearance in thin sections (Fig. 4); it appears to be built of successively layered concretions, with each layer demarcated by a ring of varying electron density. These structures vary considerably in size, with the larger ones averaging about 3–5 μm in diameter. The periphery of such granules has a spiculate appearance due to the presence of densely stained, needle-like crystals similar to those shown in Fig. 3. Presumably each concentric layer of these granules is formed by aggregation of these needles. Needle-like crystals are also present in clusters separate from the concentrically layered granules and in this case are seen in or on mitochondria, sometimes almost masking the mitochondrial profile. Since mitochondria freshly isolated from homogenates of crab hepatopancreas have been found to contain calcium and phosphate in large quantities (2), it appears possible that many of the clusters of needles actually represent deposits of calcium and phosphate present in the mitochondria of intact cells in vivo. However, the great majority of the mitochondria in sections of intact hepatopancreas cells show few, if any, electron-dense deposits in the matrix.

Cells containing the concentrically layered type III granules, in contrast to cells containing only type I and/or type II granules, frequently are damaged and show a high degree of cellular disorganization. The concentrically layered granules are only infrequently present in cells containing the other two types of structures. However, all three of these unusual mineral-containing structures described above also are seen extracellularly, usually appearing to lie within the lumen of a gland tubule. The relative importance of each type in the function of storage and/or transport of mineral is not clear at the present time (see below). However, some structural relationship between the very densely staining (type II) and the concentrically layered structures (type III) is suggested by the inset in Fig. 2. In this micrograph a large extracellular granule can be seen which contains a very dense, mineral-like core similar in appearance to those granules illustrated in Fig. 3, with concentric layers at its periphery. In addition, two smaller concentrically layered granules are shown; from the micrograph it seems that the deposits forming the layers peel off as long, irregular strands. These strands may represent the material which is apparently missing from the intracellular granules shown in Fig. 3.

Electron micrographs of thin sections of the major components found in a typical washed calcium granule fraction isolated from a hepatopancreas homogenate are shown in Figs. 5 and 6. These granules closely resemble the type III electron-dense structures seen in the intact tissue (Fig. 4). The frequent association of those type III granules with evidence of cellular breakdown and with the *absence* of the other two granule types suggests that the type III granules represent the cell component most actively involved in secreting or transporting mineral. However, all three types of structures do appear mineralized and have been seen in both the cells and the lumina of the tubular glands of this organ. Without a more systematic (and) quantitative assessment of granule distribution throughout the intact tissue, an undertaking clearly beyond the scope of the present study, it cannot be assumed that the chemical composition of the isolated granule fraction reflects quantitatively that of the mineralized portion of the intact tissue in vivo. Furthermore, the structures in the washed granule fraction, although very similar in appearance to type III granules, do not have densely staining needle-like crystals associated with their peripheries. Fragmentation of the granules during isolation and washing might well liberate material, such as lipids, particularly from the surface of the granules, which would sediment at a rate much different from that of the mineral portion of the granules. Such a selective loss of lighter material may account in part for the relatively high mineral content of the isolated granules and perhaps for the relative paucity of the types I

and II granules seen in the isolated granule fraction: the lipid content (relative to mineral) of type I granules especially might be so high that they would migrate to the supernatant lipid layer upon centrifugation. This possibility is currently under investigation.

## DISCUSSION

The result of this exploratory study of the hepatopancreas of the blue crab more firmly establishes the occurrence and nature of physiological $Ca^{2+}$ and phosphate deposits in this tissue, which is amenable to fractionation and analysis by conventional biochemical techniques. Furthermore, the feasibility of a more systematic characterization correlating morphological and chemical measurements is enhanced by the possibility of isolating as a relatively pure and undamaged fraction one of the tissue components in which insoluble mineral is stored.

Our specific findings may be compared with several other studies of mineral deposits in soft tissue of different invertebrate species. The most similar in approach are the intensive studies of von Brand and co-workers on the calcareous corpuscles formed in the epithelial cells of the gut of various cestodes (7, 8). Despite large differences in the species studied, and in the presumed role of the mineral deposits, certain similarities between their findings and ours are noteworthy: (*a*) the similar intracellular location and lamellar substructure of the mineral storage granules, (*b*) the isolation of a subcellular fraction highly enriched in these granules, fairly intact as judged by microscope appearance, (*c*) the paucity in the isolated granule fraction of protein, polysaccharide, and lipid, the organic substances most commonly associated with calcium deposits in other mineralized tissues, and (*d*) the occurrence in the granules of an appreciable amount of organic material volatilized on ashing (9).

It appears possible that findings (*c*) and (*d*) have a common basis, namely, that the calcareous granules of the cestodes and the mineral granules of the crab hepatopancreas contain small amounts of relatively low molecular weight species which interact with the mineral phase so as to inhibit its solubilization, thereby preventing equilibration of high concentrations of mineral ions, especially $Ca^{2+}$, throughout the other subcellular compartments, In this regard, the appreciable amount of ATP and ADP found in the isolated granules in the present study is of particular interest. Termine and co-workers have shown that both $Mg^{2+}$ and pyrophosphate facilitate the initial precipitation of insoluble calcium phosphate at physiological pH and ionic strength (10), and that both ions also tend to stabilize the amorphous solid phase first formed, inhibiting its transformation to crystalline hydroxyapatite (11). The considerable amounts of $Mg^{2+}$, ATP, and ADP found in the mineral granules of the crab hepatopancreas might serve to stabilize insoluble calcium phosphate, as apparently occurs in massive-loaded mitochondria (12, 13).

The only other report which offers a chemical comparison between a similar whole tissue and its mineralized component concerns the digestive gland (hepatopancreas) of the snail *Helix pomatia* (14). The $Ca^{2+}$ and $Mg^{2+}$ levels as a percentage of the whole tissue are close to those reported here for the crab hepatopancreas. The isolated "spherules" from the snail gave the molar ratios of Ca, Mg, phosphate, and carbonate as 1.0:0.6:1.0:0.1, very close to our 1.0:0.4:0.8: <0.1.

The types I and II granules found in crab

FIGURE 2 Thin section of crab hepatopancreas showing type I granules containing smaller, more densely staining inclusions. Fixed and stained as in Fig. 1. × 7,000. *Inset*: Granules within lumen of the hepatopancreas. Type III granules and a form composed of a dense core and concentric peripheral layers are seen. See text for details. Fixed and stained as in Fig. 1. × 7,000.

FIGURE 3 Thin section of hepatopancreas illustrating type II granules consisting of extremely electron-dense core with needle-like crystals at surface of granules. Nonstaining region may reflect compartpartmentation by an enclosing membrane and shrinkage, displacement, or extraction of granule. Coalescence of small vesicles may be involved as indicated by arrow. Fixed and stained as in Fig. 1. × 10,500.

FIGURE 4 Type III granules in hepatopancreas. Distinguished by concentric layers of varying electron densities and peripheral needle-like crystals. Needle-like clusters are also seen elsewhere in cytoplasm, sometimes in association with mitochondria (*m*). Disorganization of cell structures is apparent. Fixed and stained as in Fig. 1. × 7,000.

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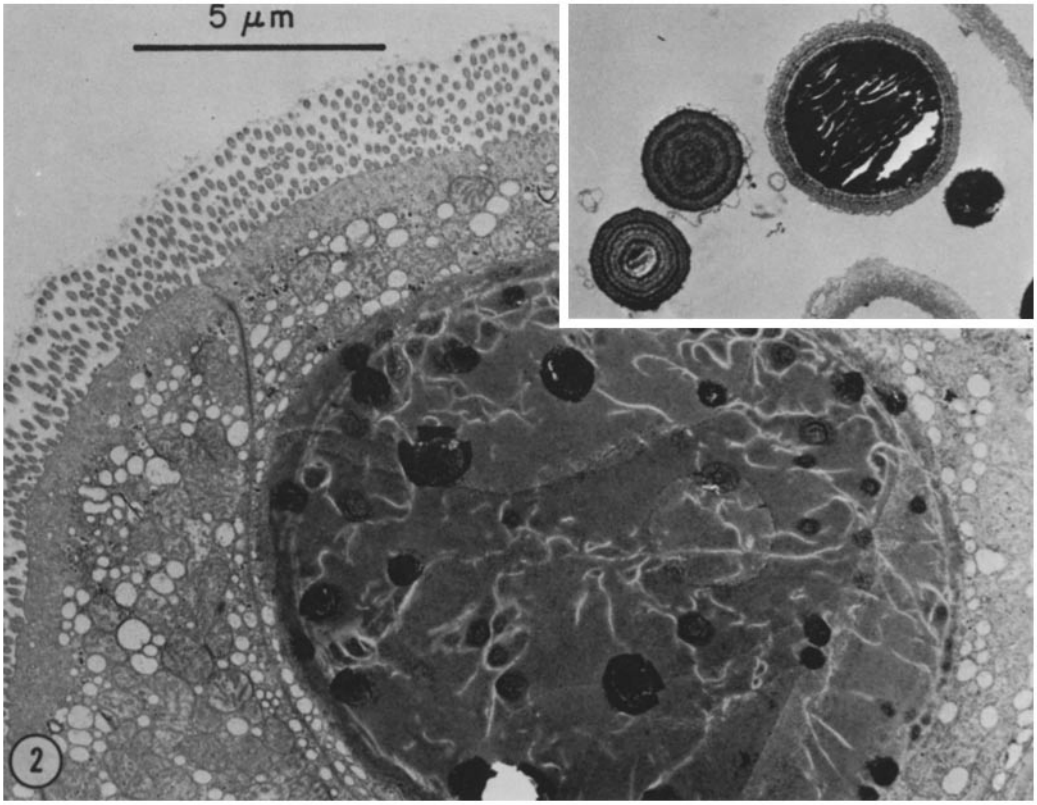


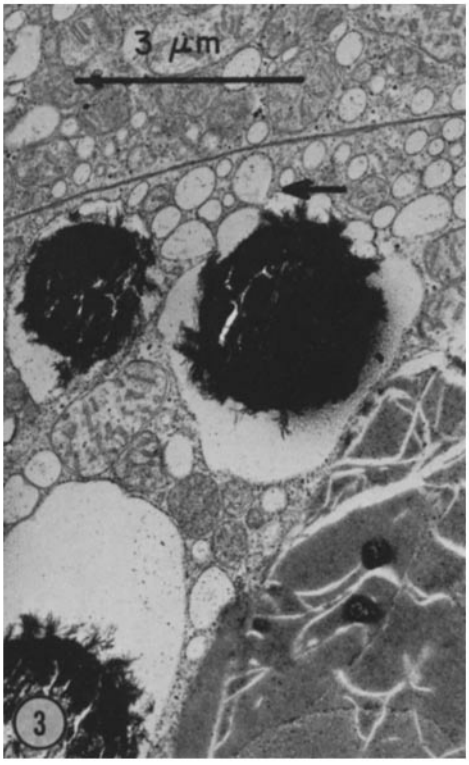


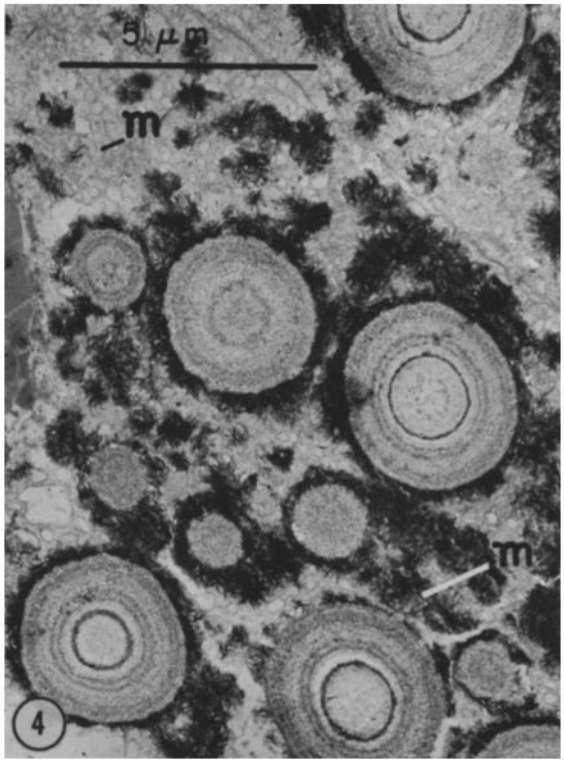

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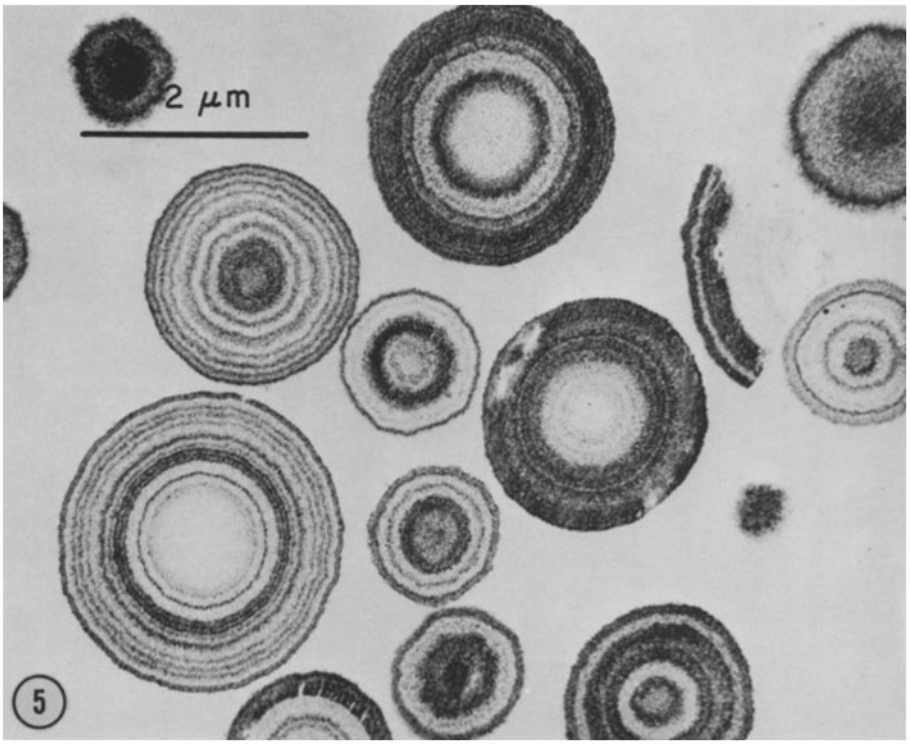


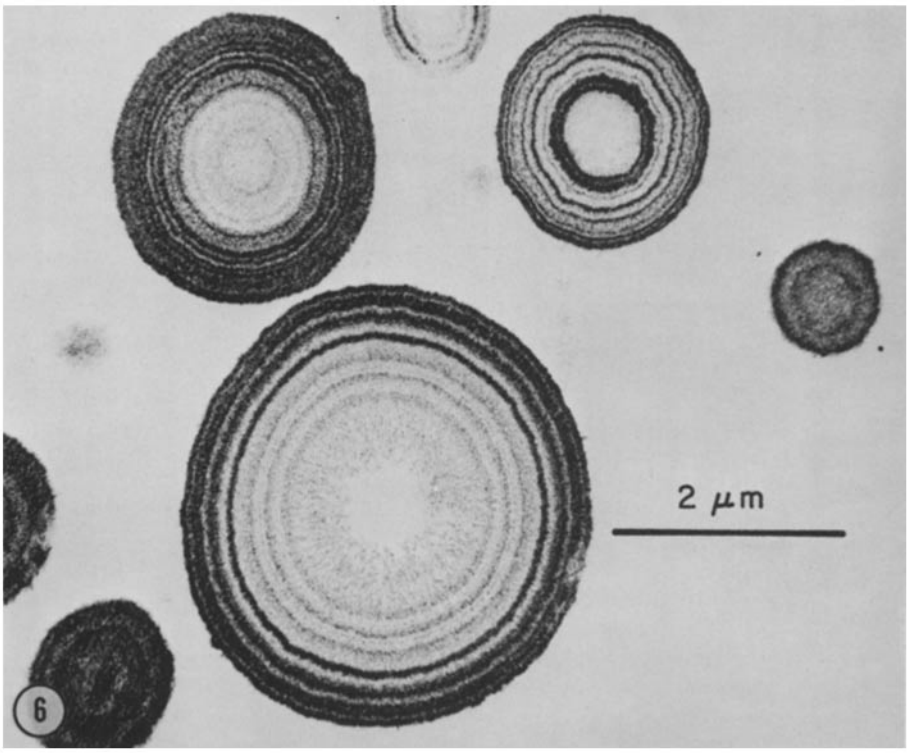


FIGURES 5 and 6 Thin sections of isolated washed granule fraction. Embedded in low-viscosity medium described by Spurr (4). Range of sizes of isolated granules similar in general appearance to type III granules is apparent. Distinct layers of varying electron density are clearly visible. Peripheral needle-like crystals of type III granules are not present. Fixed and stained as in Fig. 1. × 16,000.

hepatopancreas are morphologically unique among previously described soft tissue mineral deposits. The physiological occurrence of mineral granules in the *lumen* of tubular glands has been reported in trematodes (15), where these structures are thought to constitute an excretory form of excess Ca and Mg. While hepatopancreas cells of a variety of crustacean species have been shown to carry out apocrine, merocrine, or holocrine secretion (16), we know of no information concerning the mineral content of the secretory products. Furthermore, although mineral stored in the crab hepatopancreas is known to be returned to the exoskeleton (16), the cellular mechaism of mineral mobilization has not been characterized. On the basis of our findings, we would suggest that the extracellular, intraluminal granules which we observed could reflect a mechanism of $Ca^{2+}$ mobilization in the crab. Depending on the animal's need for mineral ions, release of these granules from the cells into the lumen could be followed by excretion of the intact granules through the gut or by reabsorption of the ions after chemical and enzymatic dissolution of the granules. Such release might well require little energy expenditure by the cell; furthermore, granule degradation could occur much more rapidly and/or under more extreme conditions in the gut than inside the epithelial cells, since in the former highly active, relatively nonspecific digestive enzymes or pH extremes could be utilized.

Intestinal degradation of aggregates of stored mineral has in fact been reported for other decapods, including most of the crayfish species. The latter retain a significant portion of their stored mineral within the walls of the foregut, in the form of extensive circumscribed mineral plaques (gastroliths). After molting has occurred, the gastroliths are sloughed into the lumen of the gut, where digestive juices solubilize the mineral ions for subsequent reabsorption (17).

A possible relationship or homology between mineral deposition in crab hepatopancreas and calcification as seen in vertebrate hard tissues seems enhanced by the recent finding that vesicular structures, presumably derived from cells, appear to be the locus of initial mineral-phase formation in epiphyseal cartilage of rabbits (18). Thus preliminary cellular events have been linked very directly to subsequent extracellular calcification.

However, there are some important apparent differences to be explained before this homology can be accepted. In the crab the granules are mineralized inside the cells of a parenchymal tissue, whereas the much smaller matrix vesicles of bone are first seen extracellularly as unmineralized, membrane-bound structures. It is possible, however, that the mineral granules formed intracellularly in the crab may at some point acquire an investing membrane before or during their extrusion from the cell. It is also possible that the homogeneous, relatively electron-dense material within apparently unmineralized cartilage vesicles (18) may represent mineral in some organically bound and/or noncrystalline form, perhaps stabilized by a mechanism similar to that postulated for the crab hepatopancreas granules. Electron microprobe analysis might prove useful in resolving this question.

Another possibility is that the mineralization in crab hepatopancreas has a direct counterpart in higher animals only in certain pathological conditions. Several authors (19–21) have proposed that kidney stones are initiated intracellularly, then extruded into the urine, where their growth proceeds spontaneously in the presence of relatively high concentrations of mineral ions. Another, more general observation is that those tissues most prone to nonspecific pathological calcification are epithelia which generate and/or maintain a transmural pH gradient, such as the kidney, stomach, pancreas, and lung. The crab hepatopancreas is a secretory gland as well as a mineral storage organ; moreover, the digestive juice that it elaborates, in those Crustacea in which it has been studied to date, appears to have a rather closely regulated pH.

The role of mitochondria in the mineralization phenomenon described in this report remains uncertain, supported by only circumstantial evidence. The preceding paper (2) confirms that the mitochondria of this tissue can load unusually large amounts of $Ca^{2+}$ and $P_i$ ions in vitro, and that the insoluble salt which forms within the matrix appears to be crystalline. Such mineral aggregates might be extruded or released from mitochondria, as postulated earlier (3) and as suggested by the morphological findings of Trump et al. (21). Crystallization of calcium phosphate in the mitochondrial matrix could cause loss of the ability of the mitochondria to regulate their internal levels of calcium and phosphate, by damage to the mitochondrial membrane. These crystalline aggregates, if exposed to ion concentrations in excess of the mineral-phase solubility

product, would tend to undergo spontaneously a further increase in size, via simple crystal growth. These processes might lead to the kind of crystals associated with mitochondria seen in Fig. 4. The possibility that such crystals might serve as a nidus around which the large (type I) cytoplasmic aggregates could form is suggested by the finding of focal areas of increased electron density within the type I granules.

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