Butll. Inst. Cat. Hist. Nat., 47 (Sec. Zool., 4): 93-103. 1981

MINERAL CONCRETIONS ON THE INTESTINAL EPITHELIUM OF CYCLOPS STRENUUS FISCH (CRUSTACEA: COPEPODA). ULTRASTRUCTURAL STUDY

Rebut: abril 1981

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RESUM

Concrecions minerals a l'epiteli intestinal de Cyclops strenuus **Fisch** (Crustacea: Copepoda). **Estudi ultrastructural**

La porció mitjana i posterior del tub digestiu de **Cyclops strenuus** té dues funcions, una d'absorbent que té lloc a nivell de les cèl·lules intestinals de tipus A i que és la funció habitual de la mucosa intestinal, i la segona, acumuladora de sals minerals, principalment calcàries, que sota la forma de cossos o concrecions esfèriques apareixen en el citoplasma de les cèl·lules de tipus B.

Els resultats obtinguts després de l'aplicació de tècniques adients de la microscòpia òptica i també de la microscòpia electrònica assenyalen que aquestes concrecions tenen una matriu orgànica, rica en mucopolisacàrids, que donen reacció Thièry positiva en la fase inicial de la seva formació, a nivell del reticle endoplasmàtic de tipus llis. És en aquesta xarxa de glucoproteïnes on es dipositen i emmagatzemen les sals calcàries: carbonats, fosfats, sulfats, etc.

Les cèl·lules de tipus B, en menor nombre que les de tipus A, tenen microvillis més curts i molt més escasos; entre aquestes formacions s'hi troben formacions de pinocitosi, encarregades de capturar les sals, les quals són seguidament canalitzades per un reticle endoplasmàtic de tipus llis i tubular, molt desenvolupat, donat que prové en gran part del reticle rugós, per pèrdua dels ribosomes.

Després de la incorporació de les sals hi ha una proliferació de formacions mielíniques, lloc on es dipositaran les sals.

Formacions similars han estat descrites en els tubs de Malpighi de diversos insectes, encara que en aquests casos el significat i la composició química de les concrecions és molt diferent: es tracta d'urats, generalment, que queden bloquejats.

En **Cyclops strenuus** l'acumulació de sals, que dóna lloc a les concrecions esfèriques, està relacionada amb el procés de calcificació del tegument després de l'ecdisi, i en aquest sentit els nostres resultats poden comparar-se amb els obtinguts per Graf en els seus estudis sobre **Orchestia** i **Niphargus**.

És a dir, després de la muda, les concrecions calcàries se solubilitzen i travessant la

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làmina basal de l'epiteli intestinal passen a l'hemolinfa i arriben a la cutícula, consolidant-la.

En els estudis ultraestructurals que hem dut a terme sobre la mucosa intestinal de copèpodes lliures i endoparàsits, cal remarcar que només hem constatat la presència de cossos calcaris a nivell de les espècies lliures; a les endoparàsites no els cal calcificar la cutícula, que per altra banda està molt simplificada, a causa del règim de vida.

INTRODUCTION

Mineral and puric bioaccumulation is proper to many groups of invertebrates and it is determined, apart from the physiologic features of each species, by diverse external limiting factors, as nutrition, salinity and temperature, among others.

The majority of cations are found in the form of carbonates, phosphates, oxalates or urates; these later are found above all in Insects (RAZET, 1961; LHONORÉ, 1976). Generally, they form intracytoplasmic spherical concretions (spherulites) of variable size, and they have been described in various cell types, although they are found above all in hepatopancreas of Mollusca and in Malpighian tubules of Arthropoda. Exceptionally, mineral concretions have been described in female germinal cells of Myriapoda (Petit, 1970) and in Lepidoptera ovocytes (Cantacuzéne, 1967, 1968).

In Insects, it has been confirmed that the intestinal epithelium acts as a selective barrier, which not only works for cations usually brought in by nutrition (Mn, Fe, Zn, etc.), but for the ones which are accidentally found there (Al, Ba, Cd, Co, Ni, Hg, Si, etc.). This fact is of considerable interest and must be taken into account when combining the elements which are part of insecticides, and we must bear in mind that Insects can cure themselves of intoxication with the possibility of accumulating toxic cations in the form of spherocrystals; in this way they leave them inactive (LHONORÉ, 1973, 1976; JEANTET *et al.* 1974; MARTOJA & MARTOJA, 1973), and so they can stand an extended treatment in perfect conditions.

Crustaceans with calcified carapace are an extremely interesting material for the study of calcium interchange, due to the fact that their exoskeleton is periodically renewed and the calcification of the new cuticle is carried out after molting, and so the loss of calcium due to exuviation is quickly compensated (GRAF, 1974).

Crustaceans cuticle is usually made up of four layers: the most external layer, the epicuticle, is lipoproteic and lacks chitin and mineral salts. The three other layers consist of an organic fibrillar matrix where chitin is fixed. In the pigmentary and the principal layer there are variable quantities of calcareous salts, depending on the species in study and also directly depending on limiting factors such as salinity, temperature, etc.

Calcium is precipitated on the cuticles in the form of calcite or in amorphous state. The proportion of phosphates with respect to carbonates is the limiting factor of this; so, when this rate is higher than 0.105 mg/l calcium is stable in amorphous state, while at lower levels of phosphate it crystallizes.

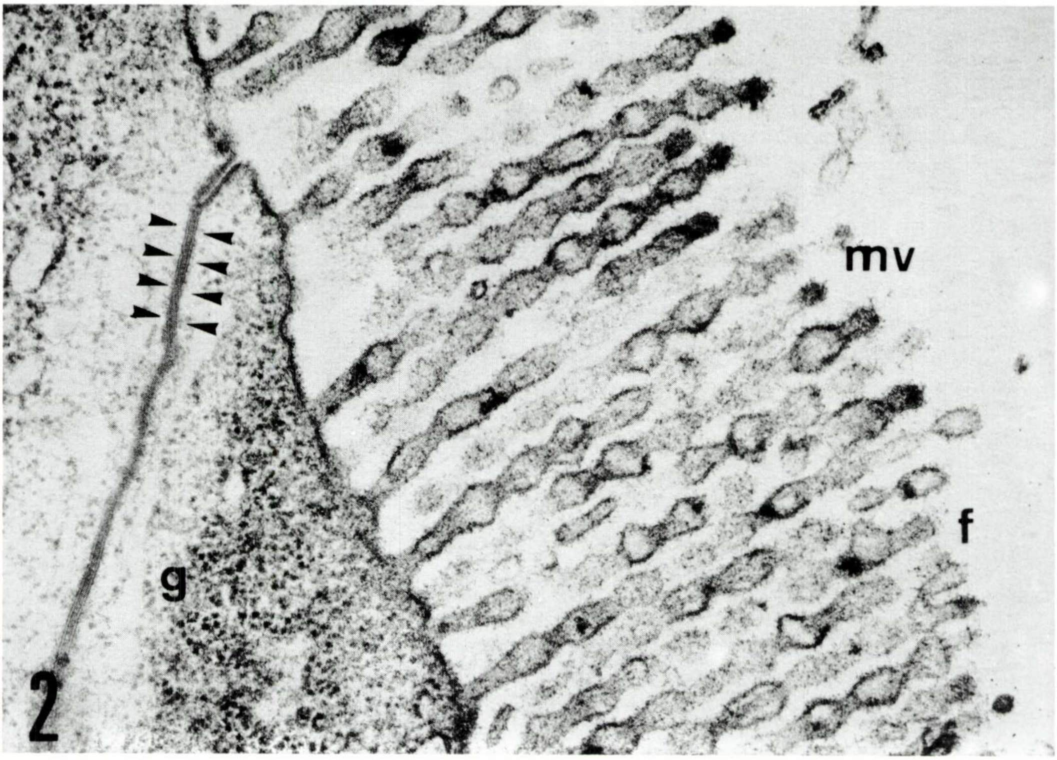
The source of integumentary calcium can be exogenous or endogenous. In the majority of species we find the two origins simultaneously.

Direct absorption of calcium from water through the integument was described for the first time by HECHT (1914) in *Callinectes* and later verified by SCHUMANN

ABREVIATIONS IN THE FIGURES

A.	type A cell
B.	type B cell
C.	calcareous concretions
ER.	endoplasmic reticulum
mv.	microvilli
N.	nucleus
n.	nucleoid concretions
f.	fuzz
g.	glycogen
p.	pynocytosis
ser.	smooth endoplasmic reticulum
vser.	hypertrofied vesicles of the smooth endoplasmic reticulum.

FIG. 1. Intestinal epithelium ultrastructure of *Cyclops strenuus*. Two type A cell and one type B cell can be observed. The arrows show tight junctions (7.500 x).
Ultraestructura de l'epiteli intestinal de *Cyclops strenuus*. S'observen els dos tipus cel·lulars que es troben en el tram mitjà de l'intestí. Les fletxes assenyalen contactes del tipus de les unions íntimes (7.500 x).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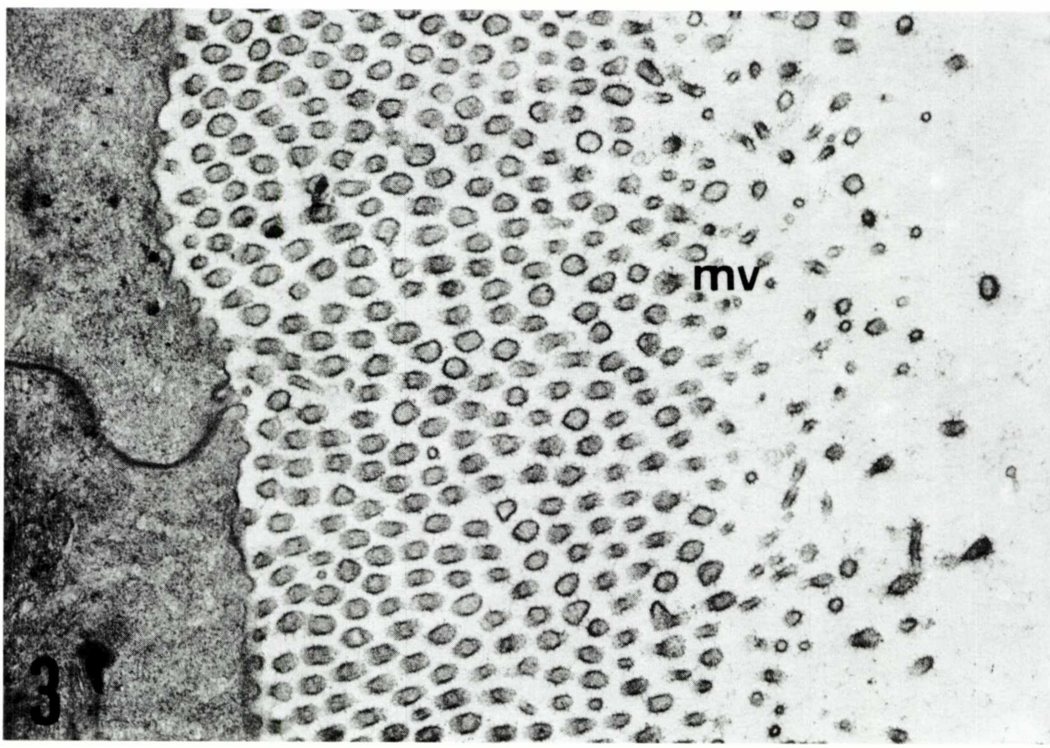
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FIG. 2. Apical pole detail in a type A cell, where sinnous microvilli can be observed (mv). Notice the intermediate junctions shown by the arrows (30.000 x).
Detall de la regió apical del tipus cel·lular A. Hom pot observar molt bé l'aspecte sinuós, arrosariat, que tenen els microvillis (mv). Les fletxes assenyalen contactes tipus *zonula occludens* (30.000 x).

(1928) in *Gammarus,* when he observed that the new cuticle did not consolidate when, after exuviation, specimens were in water tanks where the calcium content was lower than 130 mg/l.

It was confirmed that when the calcium content in sea water is about 400 mg/l there is a quick integumentary absorption, which depends directly on temperature (VINCENT, 1971). In fresh water the calcium amount varies from 0 to 140 mg/l. NYGAARD (1955) verified that *Astacus fluviatilis* consolidates its carapace in water very poor in calcium (about 1,8 mg/l). The intensity of calcium absorption varies depending on the species. For example, in *Carcinus* DRACH (1939) calculated that it was 160 mg/day and for 100 g fresh weight of the specimen.

Although it has been demonstrated that calcium is in many species directly absorbed by the integument, it must be admitted that the major part of the calcium in the exoskeleton comes from the calcium absorbed through the branchial and intestinal epithelia. After previous storage in them or in the hepatopancreas, calcareous salts are redisolved and through haemolymph they reach the integument, during the post-exuvial stage.

In this account an ultrastructural study of spherocrystals found during the pre-exuvial stage in the midgut of the fresh water copepod *Cyclops strenuus* Fisch is carried out.

FIG. 3. Cross section of type A cell microvilli; notice the high electron density of their periphery, as well as the delicate fibrillar network (fuzz) (30.000 x).
Tall transversal dels microvillis de la cèl·lula intestinal de tipus A. Es pot observar un ben contrastat glicocàlix envoltant-los *(fuzz)* (30.000 x).

MATERIAL AND METHODS

The specimens of *Cyclops strenuus* which have been studied come from the Clot d'Espolla inundation pan and Banyoles lake (Girona, Spain), a carstic and oligotrophic lake, the calcium content of which varies from 0.31 to 0.67 mg/l (PLANAS, 1973).

Entire specimens were fixed so as to study them with light and electron microscopes with usual techniques. The samples to observe by light microscopy were fixed, better in formol (8 to 10 %) or in liquid of Pampel or in glutaraldehyde (3 %).

Spherulites, the object of our study, were detected by light microscopy on histological sections 8 μm thick, which had been stained according to different techniques. Among them we could mention the use of alizarin red S (0.1 %); with it spherulites are very well observed, showing their high calcium content. We have also used titan yellow, in order to determine their possible magnesium content, and Gomori's method to identify possible urate formation, the results of which have been negative and so we reject this composition. Silver impregnation (silver nitrate 2 %) is highly demonstrative of these concretions. Also, in certain stages they give positive reaction to PAS and Hotchkiss-MacManus techniques; this makes us suppose that they contain an important charge of mucopolysaccharides.

Specimens to study by electron transmision microscopy were fixed with glutaraldehyde (3 %) in Sorensen or cacodilate buffer (pH 7.2 and temperature 4°C), usually followed by postfixation with buffered osmium tetroxide (2 %). After an accurate dehydration, specimens were embedded in Araldite-Epon or in Spurr. Ultra-thin sections were stained with uranyl-acetate and with lead citrate prepared according the Reynolds technique.

Thiery's technique (1967) has been used to detect mucopolysaccharides, giving very good results applied on growing concretions.

Ultra-thin sections have been observed with Philips EM 200 and Philips EM 301 transmission electron microscopes from the Electron Microscopy Service of the University of Barcelona.

OBSERVATIONS

In the mid and hind-gut *Cyclops strenuus* we find a mucosa which emits slender villi 60 to 80 μm long, and consists of a columnar epithelium where two cell types can be distinguished; they are morphologically and functionally different and they rest on a thin, sinuous basal lamina, which separates them from a thin circular musculature layer.

The most abundant cells in the intestinal epithelium, the ones we call type A cells, are the most slender and not typically prismatic, but rather pyramidal, forming the villus and resting on a cuboidal epithelial cell, which is going to replace the last one. Among the villi we find type B cells; they are the ones which show mineral concretions during the pre-exuvial stage.

TYPE A CELLS

They measure from 35 to 40 μm high and 12 μm diameter in their basal zone, and from 4 to 8 μm in the apex. The presence of many microvilli on their apical pole is an important feature; they are orderly distributed and show a very constant length: 2 μm, and are 0.1 μm thick (fig. 1).

These microvilli also show two interesting features: firstly, they are extremely sinuous, which is uncommon in this structure; secondly, the presence of fibrillar elements, which can be perfectly seen whatever the fixation technique used may be. Such microvilli are surrounded by a delicate glycocalix which in certain zones, mainly on the apical pole, is arranged as a «fuzz», so that a lax fibrous network is created among the microvilli and appears in cross sections (figs. 2 and 3).

A large amount of endoplasmic reticulum vesicles occupy nearly all the cytoplasm and is placed parallel or concentrically. This feature, together with the presence of abundant chondriome, made up of mitochondria with dense matrix and many cristae, allude to the synthesizing function of these cells.

We find the nucleus occupying first the basal zone, but after it moves and takes an elliptical shape; it is rich in chromatin and has abundant interchromatinic and perichromatinic granules.

FIG. 4. Apical pole detail of a type B cell. Notice the amount of pinocytosis vesicles which are formed by invagination (p), and their release (*). The smooth endoplasmic reticulum is extremely developed (ser) (28.000 x).
Detall de la regió apical d'una cèl·lula de tipus B. Entre els microvillis es poden observar formacions de pinocitosi (p) que donaran lloc a la formació de vesícules (*). El reticle endoplasmàtic llis es presenta molt desenvolupat (ser) (28.000 x).

The type A cells are replaced by others which occupy the basal zone and have cuboidal shape and all the features of the first cells, except for the lack of microvilli, due to their lack of free surface.

Intermediate junctions can be observed among type A cells, in the depressions left by the villi (figs. 1 and 2). No juntion has been seen between the basal surface of the microvilli cells and the apical surface of the substituting cell.

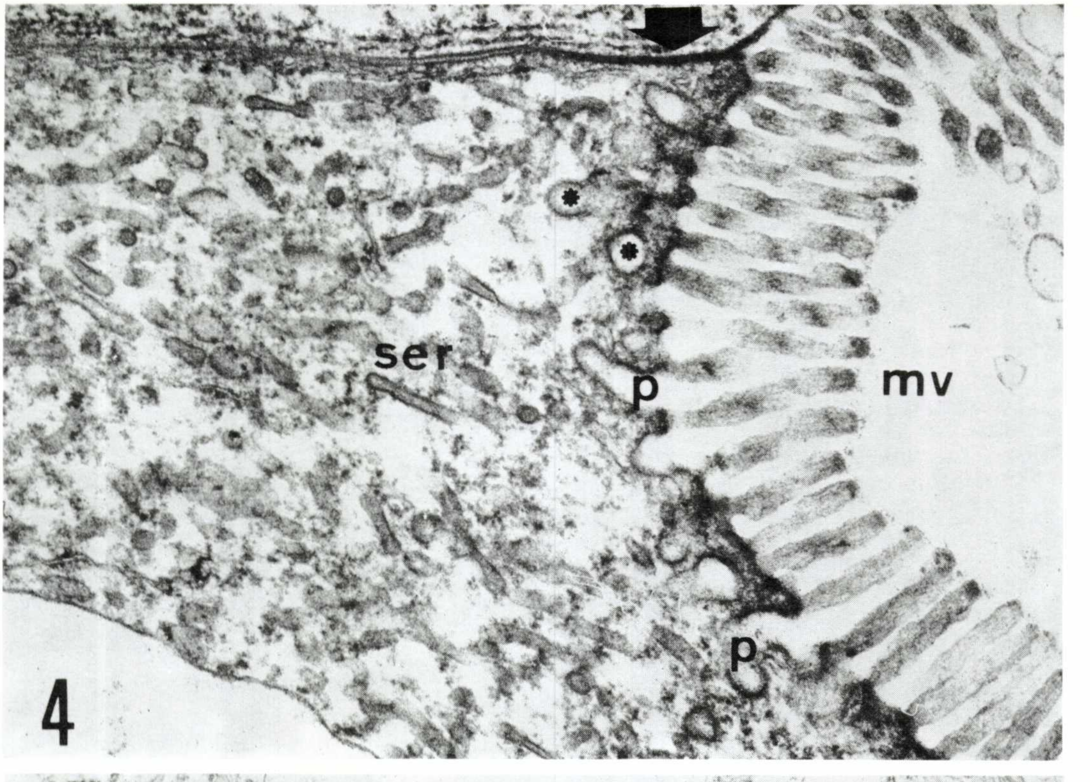
This cell type never shows any kind of mineral concretions.

TYPE B CELLS (cells with concretions)

They are the 25 % of intestinal epithelium cells. Their shape is rather cuboidal with blunt evaginations among the villi. They show on their apical pole some microvilli, shorter than the ones of type A cells, measuring from 0.5 to 0.8 μm length and 0.1 μm diameter; they are digitate and surrounded by a delicate glycocalix. It is a particularly interesting fact that very apparent endocytosis formations often occur among them, and many pinocytosis vesicles appear on the apical pole of the cell.

There are also on the apical pole of type B cells many long tubular vesicules with a high electron density, giving negative reaction to Thièry's technique. This makes us considerer them as smooth en-

FIG. 5. Type B cell with large endoplasmic reticulum vesicles (vser) where granular material is accumulated in I and II until they form concretions (C) with an organic central zone (n) (12.000 x).
Cèl·lula de tipus B amb grosses vesícules del reticle endoplasmàtic llis (vser) on s'acumula un material granulós (I i II) fins a esdevenir una concreció (C) amb una àrea central orgànica que és l'anomenat «nucleoid» (12.000 x).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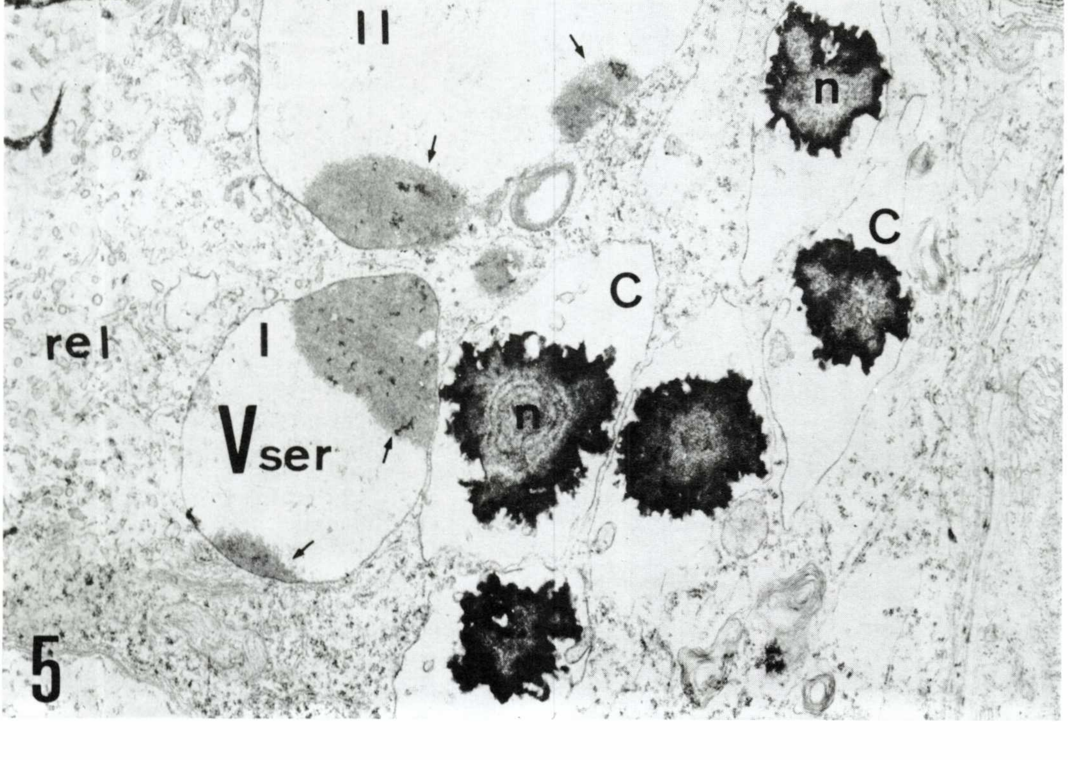
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doplasmic reticulum vesicles, which in deeper zones appear very developed and in very peculiar concentric arrangements, sometimes showing a strong polymorphism. In certain stages of the exuvial cycle, the smooth endoplasmic reticulum shows large expansions and myelination processes begin in the periphery (fig. 8).

It is in these myelinic formations where a mucopolysaccharides accumulation takes place, forming a granulous fibrous organic matrix, where calcareous salts will later deposit in order to give rise to spherocrystals, which are the reason of this study (figs. 6, 8, 9 and 13).

During the pre-exuvial stage some concretions have been found measuring from 2 to 4 μm diameter, with irregular shapes which become round as they consolidate. They show in the middle a nucleoid of not very high electron density; a good contrast is obtained applying Thièry's technique. This nucleoid slowly becomes compact and around it concentrical formations with variable density appear, observed as alternating zones which give to the spherocrystal a typical aspect (figs. 10, 11 and 12).

After exuviation we have verified how spherocrystals change their aspect and become star-shaped, extremely irregular, due to the redissolution of the salts which make up spherulites (fig. 13).

Type B cells show a well developed chondriome in the basal zone, as well as an important group of lysosomes during exuviation and post-exuviation stages.

We find tight zones and intermediate junctions between type A and type B cells (figs. 1 and 4).

GRAPHIC SUMMARY
RESUM D'IMATGES

FIG. 6. Intestinal epithelium with type A and type B cells (3.000 x).
Epiteli intestinal amb els dos tipus cel·lulars (3.000 x).

FIG. 7. Mineral salt absorption zone of a type B cell (17.000 x).
Zona d'absorció de les sals minerals en una cèl·lula de tipus B (17.000 x).

FIG. 8. Hypertrophied smooth endoplasmic reticulum with diverse myelinic formations (arrows) (15.000 x).
Vesícules hiperdesenvolupades del reticle endoplasmàtic llis, presentant formacions laminars de tipus mielínic (fletxes) (15.000 x).

FIG. 9. More advanced stage in spherocystals formation (10.000 x).
Fase més avançada en la formació de les esferes minerals (10.000 x).

FIGS. 10, 11 AND 12. Spherocrystals of calcareous salts. Notice the concentric distribution and the central nucleoid (n) (5.000 x).
Cristalls esfèrics de sals calcàries, en què es poden observar la zonació concèntrica dels dipòsits i el nucleoid orgànic en el centre (n) (5.000 x).

FIG. 13. Concretion in redissolution; it belongs to a specimen in postexuvial stage (15.000 x).
Concreció esfèrica en fase de dissolució; les sals seran posteriorment lliurades a la hemolinfa. Pertany a un exemplar en fase de postexuvia (15.000 x).

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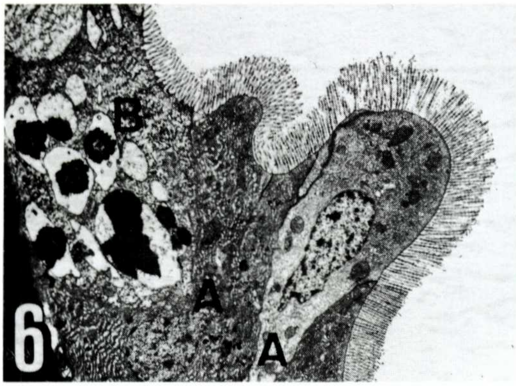
SUMMARY AND CONCLUSIONS

The intestinal epithelium of *Cyclops strenuus* has two functions: a reabsorbent one, which is carried out in the type A cells, being the usual function of gut mucosa; a second function is the accumulation of mineral salts, mainly calcareous, in spherulites placed in type B cells.

The results of the applied techniques in light microscopy as well as in electron microscopy show that type B cells concretions have an organic matrix of mucopolysaccharides, easily visible using Thièry's technique during the first stages of their formation in smooth endoplasmic reticulum vesicles. At the same time, concretions show a high calcium content. However, if more precise cytochemical methods and X-ray spectrography analysis are applied, it will be possible to determine with precision the qualitative and quantitative composition of *Cyclops* spherulites.

Our observations and images obtained allow us to affirm that mineral salts accumulate, after their absorption through type B cells microvilli, in large smooth endoplasmic reticulum vesicles, which become tubular and labyrinthic when placed near the apical pole; smooth endoplasmic reticulum canalizes dissolved salts to deeper cytoplasmic zones.

We could verify in consulted bibliography that the major part of cytoplasmic structures may be involved in the formation of different kinds of spherocrystals: from Golgi apparatus in the hepatopancreas of *Helix* (ABOLINS-KROGIS, 1970) to mitochondria, as for example in accumules of urates which appear in Malpighian tubules of *Rhodnius* (WIGGLESWORTH, *et*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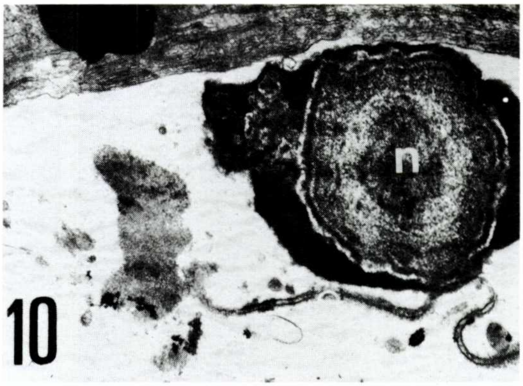
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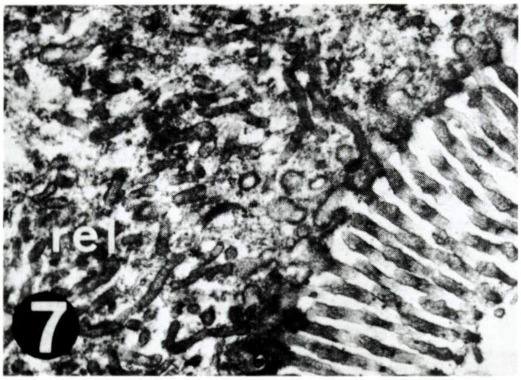
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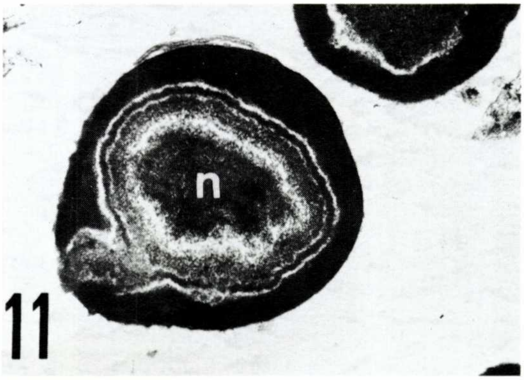
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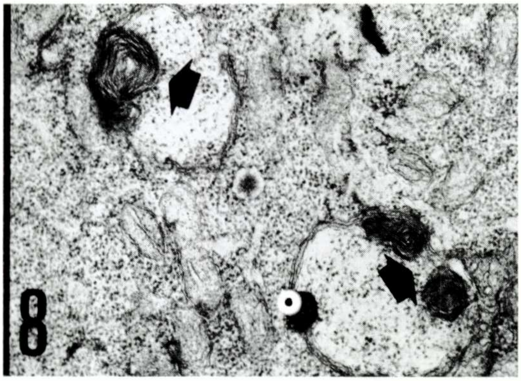
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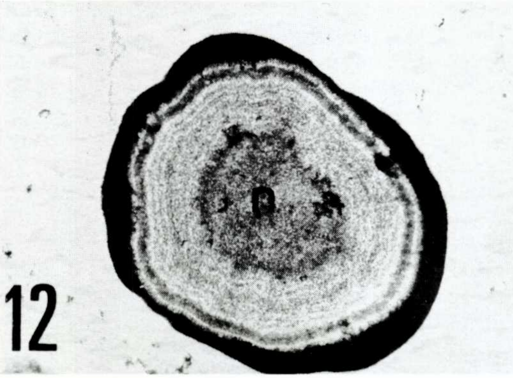
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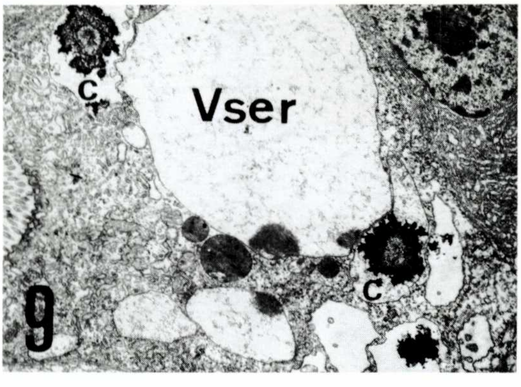
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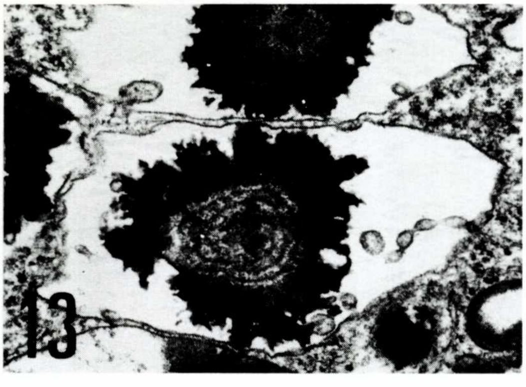
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al., 1962). Endoplasmic reticulum is, however, the most frequently involved structure in organic and inorganic salt bioaccumulation; in *Prodon* (ANDRÉ *et al.*, 1957) and in various groups of insects (BALLAN-DUFRANÇAIS, 1970, 1971) some formations have been described having this origin.

Spherocrystal formation from smooth endoplasmic reticulum has been described in Malpighian tubules of Gryllotalpida by LHONORÉ (1971), who obtains similar images to ours, although they have another meaning, because it is in this way that metabolism residual urates and phosphates are blocked.

Calcareous little spheres similar to the ones of *Cyclops strenuus*, but with different meaning, have been described in different species of Ciliates (CARASSO, 1966; FAURÉ-FREMIET *et al.*, 1968).

In *Cyclops* mineral concretions have not an excretor meaning as the ones described by DONADEY (1966, 1969) in the hind-gut of diverse groups of Crustaceans, but they have an important role in tegument calcification processes after molting; these results are the same as the ones obtained by GRAF (1974, 1978) in various studies on Crustaceans.

It is in *Orchestia* where GRAF (1969, 1971, 1977) verifies that calcium from endogenous origin is accumulated in posterior blind-guts in the form of spherical concretions during the pre-exuvial stage, which lasts about ten days. After exuviation the large calcareous concretions are solubilized in 24 to 26 hours and salts will integrate in the fibrillar organic network of the cuticle.

In endoparasitic crustaceans the integument is reduced to two layers: epicuticle and endocuticle, without traces of calcification in any of them. We do not find in these crustaceans any mineral concretions in the intestinal epithelium, nor in other organs (DURFORT, 1975, 1976, 1977).

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