Intranuclear Crystals in the Intestinal Epithelium of the Snail *Marisa cornuarietis* (Prosobranchia)

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Intranuclear Crystals, *Marisa*

Filamentous intranuclear crystals consisting of two sets of intersecting sheets are common in the intestinal epithelium. They are closely associated with chromatin granules but electron microscope cytochemistry using a modified Feulgen procedure did not show DNA in the crystal itself. Light microscope histochemical tests indicated that the crystals consist of protein.

Introduction

Intranuclear bodies or inclusions of various nature have been reported from a number of different cell types. Thus, spherical intranuclear structures were described from the nuclei of tumor cells, vascular cells and occasionally from normal tissues [1]. Rod-shaped inclusions made of arrays of 10 nm fibrillae were observed in the nuclei of cells from certain tumors [1]. An intranuclear rodlet consisting of a compact bundle of fibrils 5–7 nm in diameter was also described in certain nerve cells [2]. In molluscs, intranuclear paracrystalline structures were demonstrated in Leydig cells of *Helix* [3].

The intranuclear crystals under investigation in the present study had been demonstrated for the first time in light microscopic preparations of the ciliated mid-intestinal cells of the prosobranch *Marisa cornuarietis* [4]. These bodies were designated "nuclear filaments" and were later found to exist in nuclei of epithelial cells of different other regions of the alimentary tract of the same snail. Subsequent electron microscopic studies [5, 6] confirmed that the "filaments" are definitely intranuclear and revealed only certain peculiarities including the presence of an outer "pellicle" and the existence around them of a chromatin-free zone of the nucleoplasm. However, the thickness of the sections obtained and the magnification employed did not allow the revelation of the macromolecular organization of the "filaments".

In a latter attempt to gain an insight into the chemical nature of the "filaments" [7] a series of light microscopic histochemical tests for DNA were applied to squash preparations of the intestinal epithelial cells of *Marisa.* All three tests employed gave positive results in association with the filaments. However, these results may be taken to indicate one of two possibilities: either that the "filaments" are actually composed, at least in part, of DNA, or that the positive reaction for DNA was due to chromatin present in the immediate vicinity of the "filaments".

The present study was carried out at much higher electron microscope magnification and better resolution in an attempt to reveal the macromolecular organisation of the "filaments" or crystals as indeed they were revealed to be. Furthermore, a series of more sensitive and specific cytochemical tests were carried out at electron microscopic and light microscopic levels to try to reveal the chemical nature of these crystals.

Material and Methods

The snails were kept in aquaria at 26 °C and fed with "tetramin" fish food. Tissues were fixed for electron microscopy with 2.5% glutaraldehyde either in 0.1 M phosphate buffer or 0.1 M cacodylate buffer pH 7.2 for 1 hour at room temperature, washed in buffer and either embedded directly in Epon or after a second 1 hour fixation in 1% buffered OsO_4. Bernhard's [8] procedure for the preferential destaining of chromatin was applied to thin sections of the mid-intestine fixed in glutaraldehyde alone. Sections were treated with a concentrated aqueous solution of uranyl acetate (20 min), washed and exposed to 0.1 M EDTA for 5, 10, 20 and 40 minutes at 30 °C and poststained after washing with lead citrate (5 min). Controls were treated with distilled water. Moyne's [9] method for the cytochemical location of DNA in the electron microscope was employed with tissue fixed for 1 hour at 4 °C with 1.6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). After hydrolysis in 1 N HCl at 60 °C (10 min) and rinsing in cold buffer the tissue was dehydrated in acetone, impregnated with pyridine

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and acetylated at 45 °C in a mixture of pyridine and acetic anhydride as described by Moyne [9]. After Epon embedding and sectioning, the grids were floated face down for 30 minutes on Schiff's reagent prepared from pararosaniline hydrochloride (Merck). After washing, they were stained with thallium ethylate, again as described by Moyne [9], and examined in a Jeol JEM 100 B electron microscope at 80 kV. In controls, the treatment with Schiff's reagent was omitted.

For standard cytochemical tests on paraffin sections, the tissue was fixed in a 3 : 1 mixture of ethanol and acetic acid for the PAS-method for polysaccharides, the Feulgen reaction for DNA and for double staining with methyl green and pyronin (RNA and DNA) and in 4% neutral formaldehyde in all staining methods for proteins. Fast green at pH 2 was used to stain total protein. Basic proteins, presumably of the histone type, were stained by the method of Alfert and Geschwind, the picrid acid/eosin and the picrid acid/bromphenol blue procedure of Bloch and Hew [10].

Results

The intranuclear crystals are long, often slightly bent filaments (Fig. 5) of 0.2–0.4 µm width. Cross sections with a more or less square or rectangular outline reveal a grid-like appearance with two sets of criss-crossing diagonally arranged double lines (Fig. 1). The latter have a total thickness of about 6.6 nm. The clear space between the lines is at the limit of resolution and ought to be around 1.6 nm, each dark line measuring about 2.5 nm. The double lines are arranged with a periodicity of about 13 nm which leaves a clear space of approximately 6.4 nm between the double lines. At the edge of the crystal, the double lines are seen to bend around leading to an appearance like an interwoven mat. Crystals cut in the direction of the diagonal lines are lozenge-shaped and exhibit parallel double lines (Fig. 2) while oblique sections cut through the faces of the crystal show no very distinct line pattern (Fig. 3). It can therefore be concluded that the crystals are built up of two sets of intersecting diagonally arranged sheets. The direction of the sheets within the crystal seems to be constant since we observed no change of orientation in about 40 serial sections of the same crystal. Often, crystals are surrounded by a clear space which may show some apparently fibrillar structures (Fig. 1). Fig. 2 shows an oblique section through the end of a crystal. Crystals have been examined both after the conventional double fixation with glutaraldehyde followed by osmium tetroxide and after aldehyde fixation alone. The fine structure and the contrast are the same after both fixation methods.

We have examined snails of both sexes and of various ages (0.4, 1.2 and 2.3 cm shell diameter) and found crystals in all of them with about the same frequency. Although most frequent in the mid-intestine, they are also found in the pro-intestine, but always in the epithelium. In a few cases, we found crystals in the nuclei of glandular cells of the intestinal epithelium and not only in the non-glandular regular epithelial cells. An examination of the ctenidial epithelium of the gill revealed the presence of crystals in some of the gland cells there also. We attempted to get some information about the chemical nature of the crystals by the application of histochemical staining techniques for DNA, RNA, polysaccharides and proteins. Of these, only fast green at low pH gave a clearly positive result (Fig. 5). At pH 2, this dye is expected to stain all proteins. The standard procedure for histones using fast green at high pH gave negative results, but the bromphenol blue and eosin staining after picric acid hydrolysis was positive. There was no indication of polysaccharides using the periodic acid Schiff test and of RNA with methyl green/pyronin. Staining of DNA with the Feulgen procedure was equivocal. Due to the small width of the crystals it was not clear whether the dye was bound to chromatin close to the crystals or to the crystals themselves. Therefore we applied two histochemical tests at the electron microscope level. After 10 minutes treatment with EDTA following Bernhard's procedure [8] the crystals appeared bleached like the chromatin while RNA-containing structures like the ribosomes retained their contrast. However, the Feulgen test adapted for electron microscopy [9] showed all chromatin in high contrast while the crystals had the same contrast as the nucleoplasmic and cytoplasmic background (Fig. 4). Since this procedure is far more specific for DNA than Bernhard's regressive method, the crystals themselves do not seem to contain any DNA and the staining observed in the light microscope must be due to the chromatin particles close to the filamentous crystals.

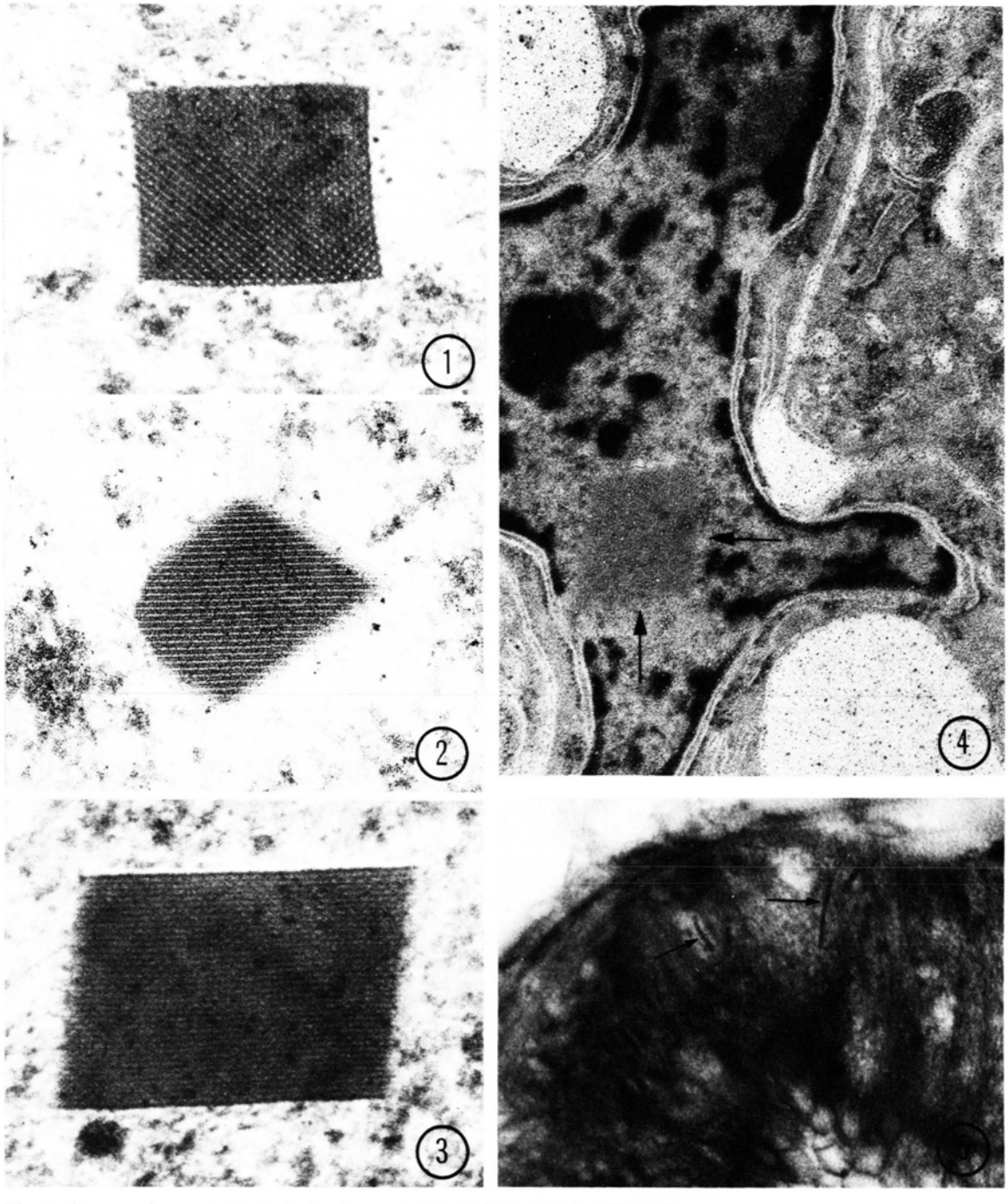

Fig. 1. Cross section; note fibrils in the clear space around the crystal. × 118 000.

Fig. 2. Oblique section, passes at the left through the end of the crystal. × 117 000.

Fig. 3. Tangential section. × 129 000.

Fig. 4. Feulgen-type reaction after Moyne; note the dense chromatin bodies and the lack of contrast in the crystal (arrows). × 60 000.

Fig. 5. Fast green FCF at pH 2. Note the filamentous appearance of crystals. × 1450.

Discussion

There have been sporadic reports about non-viral crystalline inclusions in the nucleus both from plant and animal species. Whenever histochemical studies were performed, the intranuclear crystals have been shown to consist of protein. In the case of the flour beetle, the crystals have been isolated from midgut cells and their gross amino acid composition has been determined [11]. Electron microscope autoradiography using tritiated lysine has shown that both in the flour beetle [12] and in *Gyrinus marinus*, a whirligig beetle [13], the protein is synthesized in the cytoplasm of midgut epithelial cells and passed into the nucleus where the crystals are assembled. While most intranuclear protein crystals encountered in insects are found in the epithelium of differentiated midgut cells, a crystalloid body has also been described from both spermatocytes and oocytes of a mecopteran insect at specific stages of meiosis [14].

Our histochemical staining with fast green at low pH is also indicative of a protein nature of the crystals from the mid intestine of *Marisa*. The relatively weak staining with picric acid/eosin and bromphenol blue may be due to basic amino acids. On the other hand, fast green staining at pH 8 with or without pretreatment with hot trichloracetic acid as used in the histone test was always negative. Thus, we do not expect the iso-electric point of the protein composing the crystals to be far on the alkaline side. The crystals observed in *Tenebrio molitor* consisted definitely of an acidic protein. The Feulgen reaction for DNA seemed to show linear aggregates of small granules in *Marisa* rather than a smooth filament as seen with fast green at low pH. Therefore, we thought it possible that chromatin material surrounding the filament had been stained rather than the crystal itself. The results obtained previously by light microscopic techniques [7] should be interpreted on the same basis. The Feulgen-type reaction after Moyne [9] confirmed this although the destaining of crystals pretreated with uranyl acetate in EDTA was similar to that of chromatin. However, this method [8] is not specific for DNA as such.

The shape and ultrastructure of intranuclear crystals varies from organism to organism. In dog liver parenchymal cells, they are composed of rods in hexagonal arrangement with a periodicity of 12 nm and predominant edge angles of 60° and 120° [15]. In midgut cells of the whirligig beetle the crystals are polymorphic and occur as macles [13]. Depending upon the direction of sectioning, parallel lines or crossed lines spaced 8–9 nm apart are noticed. In the epithelium of the flour beetle midgut most of the crystals are rhomboedric platelets, limited by two parallel bases. They consist of plates stacked with a 9 nm periodicity. Exceptionally, they may assume the shape of thin foils [11]. The crystals found in *Marisa cornuarietis* are thin filaments in the light microscope which may traverse a large part of the elongated nuclei. The electron microscope shows square or rectangular cross sections. The "filament" thus has the shape of a four-sided column. In contrast to the intranuclear crystals mentioned above, they seem to consist of two intersecting sets of what appear to be diagonally arranged double sheets. Thus, when the plane of sectioning is inclined with respect to the axis of the filament and when it passes at right angles to one set of sheets, the nearly square outline seen in cross sections is distorted into a lozenge shape. In this case, only one set of diagonal double lines appears since the second set is now cut obliquely and does not show up. Sections which are cut obliquely and parallel to one of the sides of the column show neither set of the diagonal criss-cross pattern clearly because both sets of sheets are inclined towards the plane of sectioning. The clear space surrounding the crystals may indicate that material has been added from the surrounding nucleoplasm during crystal growth. The apparently fibrillar structures often seen in the clear space may represent such material. If so, the sheets in the crystals may actually be built up of tightly packed fibrils.

With the possible exception of *Panorpa* gametocytes where the crystals appear at definite stages of meiosis and would thus be expected to fulfill a definite function [14], the meaning of proteinaceous crystalline inclusions in other tissues remains enigmatic. From the fact that they are not only found in mid intestine cells of *Marisa* but even in the gill epithelium we would not expect any tissue-specific function. Their appearance also seems to be independent of the age of the snails. In the midgut of the whirligig beetle [13] and the flour beetle [11] they are present only in differentiated cells and absent from the regeneration crypts. Since definite regeneration crypts are absent in the snail, we

cannot state whether the crystals are to be regarded as a sign of cellular aging or differentiation. Paraffin sections through the mid intestine show clearly that not every nucleus has a crystal. Rather, cells with crystals are less abundant than cells without crystals. Both appear intermingled in the tissue.

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