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Observations on Crystalloid Bodies in the Pseudocoelom of *Eutobrilus heptapapillatus*[1]

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Abstract: Eutobrilus heptapapillatus, found in a number of different sites at the mouth of the River Murray in South Australia, were examined under light and electron microscopes. The pseudocoeloms of these nematodes often contained oval crystalloid bodies containing carbohydrate, sulfur, phosphorus, and lipid. The bodies varied considerably in size up to a maximum of 10 μm long. The precise function of these crytalloids remains unknown. Nematodes having these crystalloids often also contained numerous small regular densely staining particles, about 20 nm d and occurring throughout the nematode's body.

Key words: carbohydrate, crystalloid body, *Eutobrilus heptapapillatus,* lipid, pseudocoelom, ultrastructure.

Eutobrilus heptapapillatus (Joubert & Heyns, 1979) Tsalolikhin, 1981 is a cosmopolitan nematode first described from stagnant water in the Tsitsikama Forest and Coastal National Park in Cape Province, South Africa (6,10). It is probably a common inhabitant of freshwater habitats throughout Australia and may prove, together with other tobrilids, to be a useful indicator of environmental pollution (1).

In many instances *E. heptapapillatus* is the predominant nematode existing in the soil at the water's edge or under fresh water at the mouth of the River Murray. For instance in a sample dredged from the bottom (3 m deep) in the middle of Lake Alexandrina, through which the Murray flows, larvae and adults of this species comprised 73% of nematodes in the sample.

In the course of an ecological study of free-living aquatic nematodes from seven sites, two estuarine and five fresh water, at the mouth of the River Murray in South Australia (Nicholas, Bird, Beech, and Stewart, unpubl.), we noticed that *Eutobrilus heptapapillatus* (6,10) occurred in each site. Many of these nematodes contained large numbers of crystalloid bodies similar to those described by Nuss (8) in the somatic muscles of *Tobrilus gracilis.* Subsequent analysis (9) with a combination of energy dispersive X-ray microanalysis and Auger electron spectroscopy indicated that these crystalloids had a high sulfur content. Nuss concluded from his studies that the crystalloids in *T. gracilis* were part of its detoxification system for sulfide ions.

Sulfur-containing crystals and crystalloids have been observed in various other nematodes such as *Haemonchus contortus,* where they are located in the intestinal cells, and *Ostertagia ostertagi,* where they are located in the intestinal lumen (2). The intestinal cells of the marine nematodes *Sabatieria wieseri, Terschellingia longicaudata,* and *Sphaerolaimus papillatus* also contain sulfur-containing inclusions (7).

In this paper we describe the ultrastructure of *E. heptapapillatus* in relation to the crystalloid bodies, report on the chemistry of these crystalloids determined by means of histochemical stains and energy dispersive X-ray analyses, and discuss their possible function.

Materials and Methods

Specimens of *E. heptapapillatus* were obtained at regular monthly intervals throughout the year from sieved sand mostly taken from the water's edge at a site above the Mundoo barrage on the southeast shore of Hindmarsh Island, which lies

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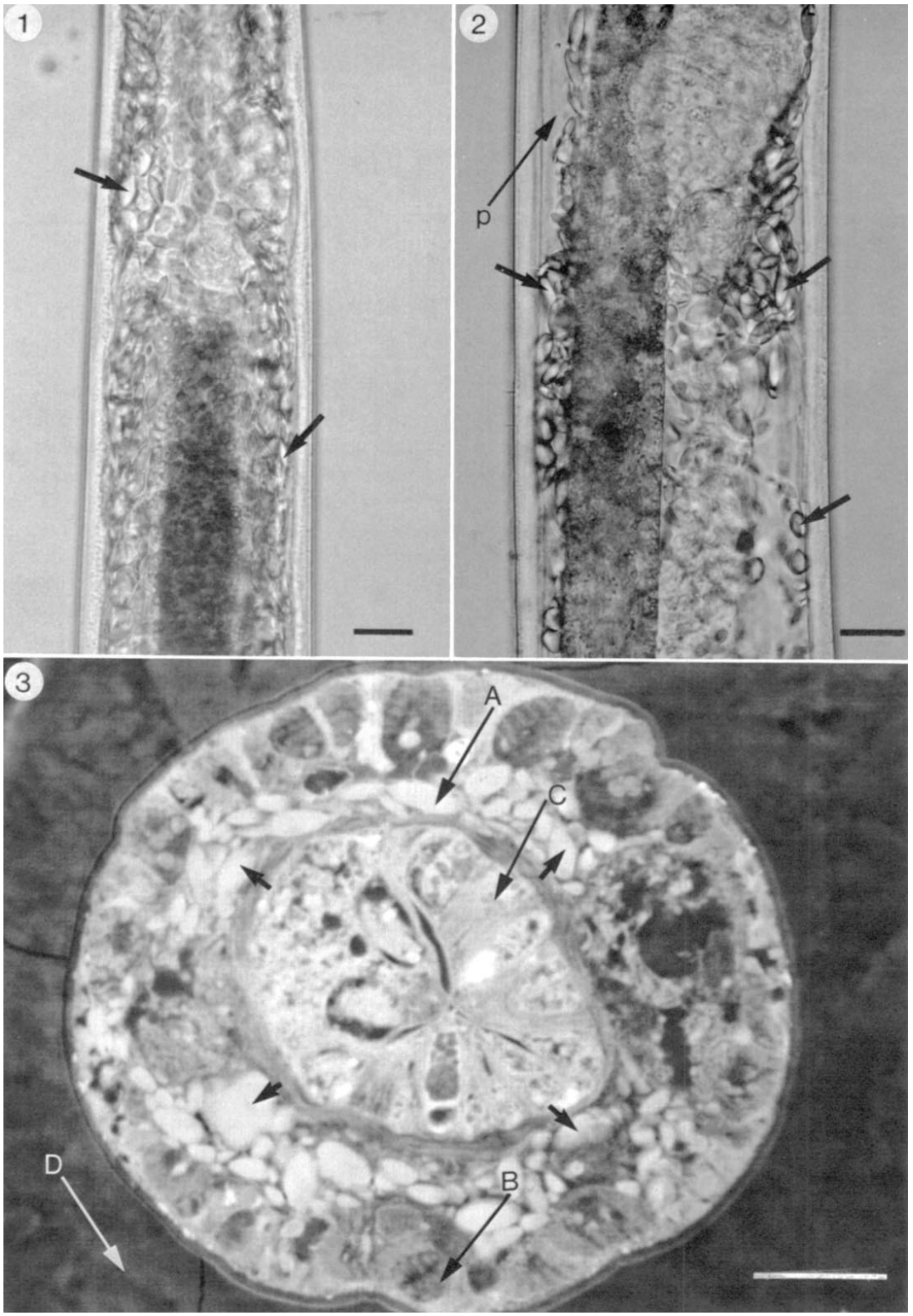
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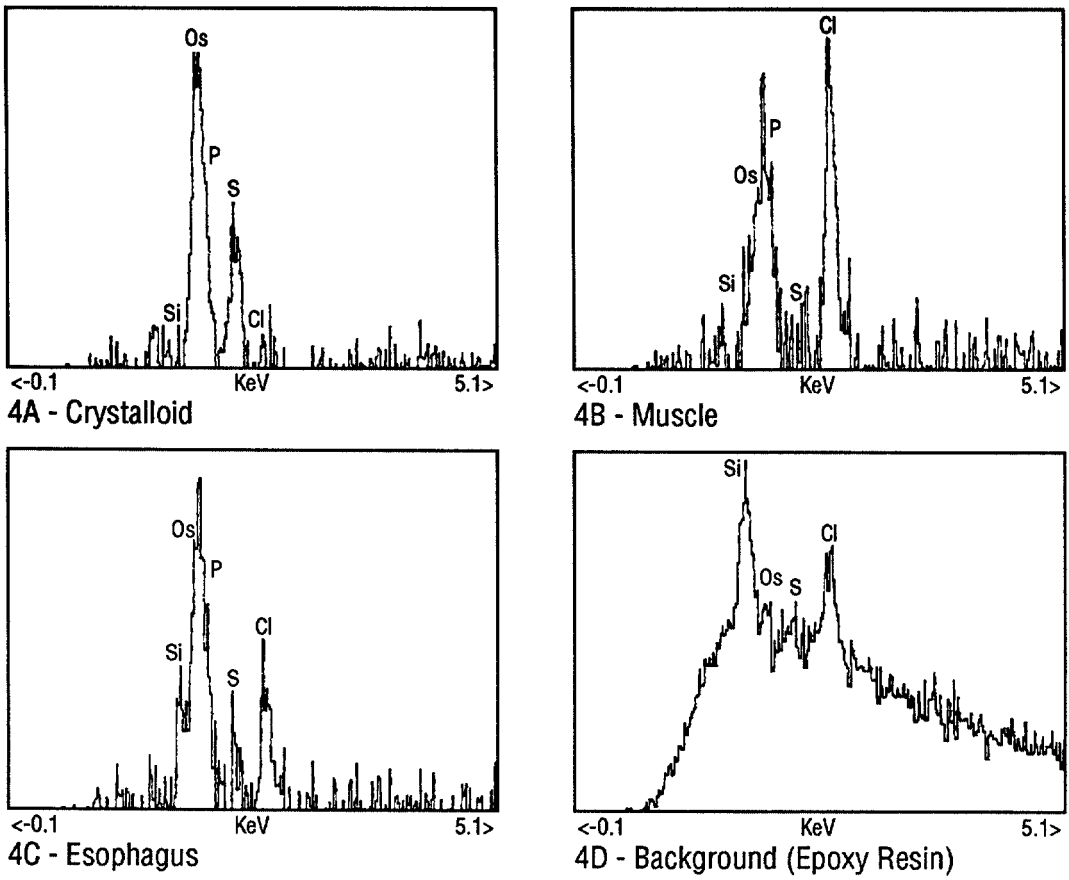

FIG. 4. EDX spectra at the various sites shown on the cross-section of *E. heptapapillatus* shown in Figure 3. A) A crystalloid. B) Somatic muscle. C) Part of esophagus. D) Background resin.

at the mouth of the River Murray in South Australia. Fixed material in glycerol was obtained from South Africa and was processed, sectioned, and observed under the electron microscope.

Light microscopy: Nematodes were photographed alive in sealed slides after they had been immobilized by anoxia. They were photographed under transmitted light using an Olympus Vanox AHBT microscope with either bright field or differential interference contrast optics. Specimens were also examined under blue and UV incident light using the AH2-RFL fluorescence attachment.

Histochemistry: Protein was stained with freshly prepared brilliant blue G (BBG, Sigma Chemical Co., St. Louis, MO) as follows: A concentrated solution of the dye in distilled water was stirred with a magnetic flea for 1 hour at 23 C and then centrifuged at 1,000 *g* for 15 minutes to re-

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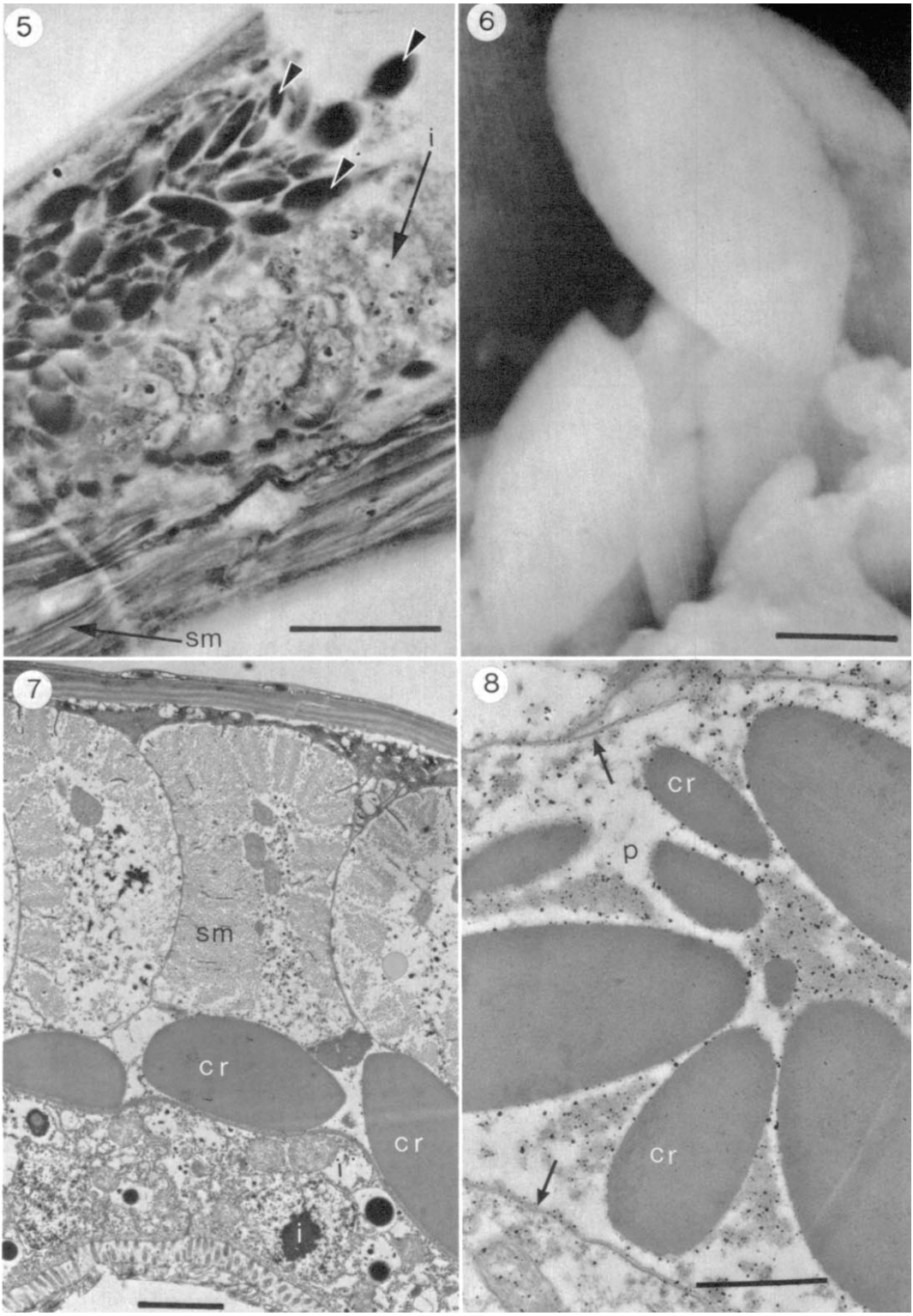
FIGS. 1–3. Crystalloids in pseudocoelom. 1) Crystalloids (arrows) packed in pseudocoelom around the junction between esophagus and intestine of a living specimen of *Eutobrilus heptapapillatus*. Scale bar = 20 μm. 2) Crystalloids (arrows) in the mid region of a living specimen of *E. heptapapillatus*, which has been slightly flattened between cover-slip and slide to illustrate the positions of these crystalloids in the pseudocoelom (p). Scale bar = 20 μm. 3) A cross section of *E. heptapapillatus*, cut through the esophageal region and viewed under the SEM, showing the crystalloids (arrows) in the pseudocoelom between the somatic muscles and the esophagus. A, B, C. D. = points where EDX spectra were collected. (See Figure 4.) Scale bar = 10 μm.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move any undissolved stain. The supernatant was sucked into a hypodermic syringe and passed through a 0.2-μm pore filter, a drop of this solution was added to 10 ml distilled water, and this solution was used for staining. Carbohydrate was stained with the periodic acid-Schiff technique (PAS) as follows: The material to be stained was oxidized in freshly made 1% periodic acid for 10 minutes, washed in distilled water, stained in Schiff's reagent (BDH Chemicals Ltd., Poole, England) for 10 minutes, washed, and mounted in distilled water for examination.

Electron microscopy: Specimens were fixed at 5 C in either 4% formaldehyde in water from their environment, which had been filtered through a 0.2-μm membrane filter, or in 4% phosphate buffered (0.1 M, pH 7.3) paraformaldehyde. Material to be examined under the transmission electron miscroscope (TEM) was left overnight at 5 C and then for a further day at room temperature (22 C) in these fixatives before being cut into several pieces with a sharp scalpel. These pieces were then washed several times in distilled water and fixed in 1% osmium tetroxide for 2 hours at 22 C, washed in water overnight, taken through an ascending ethanol series into absolute ethanol, a 1:1 mixture of absolute ethanol: propylene oxide, pure propylene oxide, and then a 1:1 mixture of propylene oxide and either TAAB 812 or TK3 resins (TAAB Laboratories Equipment Ltd., Aldermaston, England). This was followed by pure resin in vacuo at 22 C overnight. The nematode pieces were then placed in moulds in fresh resin and polymerized at 60 C. Material mounted in glycerol on slides as permanent mounts was carefully removed under glycerol and then rehydrated in a series of steps prior to fixation in osmium tetroxide and subsequent ethanolic dehydration and embedding as described.

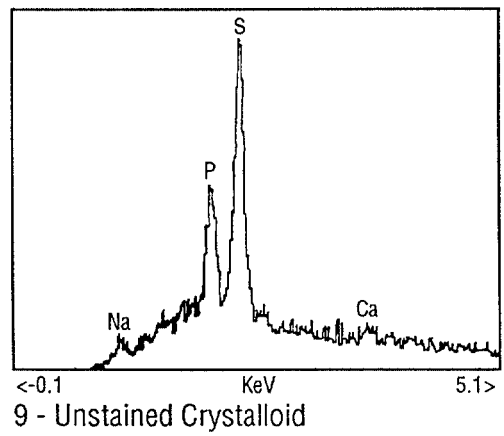

FIG. 9. EDX spectra of the crystalloids shown in Figure 6.

Sections were cut with glass or diamond knives in a Reichert-Jung Ultracut E ultramicrotome. These sections were stained in freshly made aqueous 2% uranyl acetate for 30 minutes at 22 C, washed six times in distilled water filtered through a 0.2-μm membrane filter, stained in freshly made 0.5% lead citrate in 0.1 M solution of sodium hydroxide for 10 minutes at 22 C, and washed six times in distilled water filtered through a 0.2-μm membrane filter. Sections were then examined and photographed in a Philips EM 400 TEM at 80 kV. Thick (1–5 μm) sections of this embedded material were mounted on spectrographically pure polished carbon stubs and coated with 20 nm of carbon prior to energy dispersive X-ray (EDX) analyses with a Link EDX system attached to a Cambridge S 250 Mk3 scanning electron mi-

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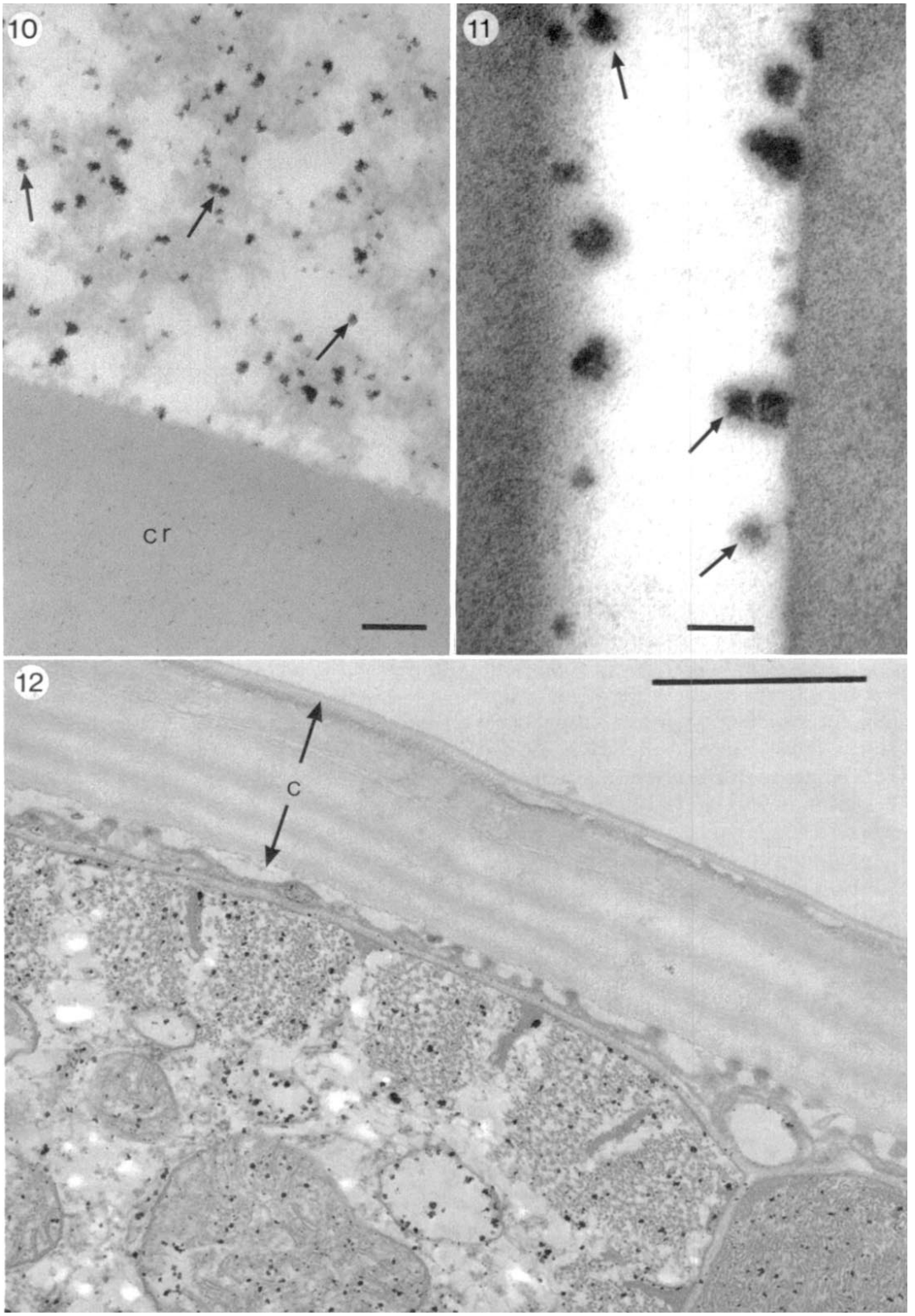
FIGS. 5–8 Crystalloids prepared in different ways. 5) Inverted back scatter electron image of a longitudinal tangential section of *E. heptapapillatus* showing crystalloids (arrows) extruded from the cut surface of the nematode and surface tangential sections of the intestinal cells (i) and somatic muscles (sm). Scale bar = 20 μm. 6) SEM micrograph of extruded crystalloids from cut *E. heptapapillatus*, not embedded and fixed only in formaldehyde. (See Fig. 9.) Scale bar = 2 μm. 7) TEM micrograph of a cross section through *E. heptapapillatus* showing the crystalloids (cr) in the pseudocoelom between the somatic muscles (sm) and the intestine (i). Scale bar = 2 μm. 8) TEM micrograph of a cross section through *E. heptapapillatus*, showing the crystalloids (cr) in the pseudocoelom (p) between the muscle and intestinal cell membranes (arrows). Note the small regular particles visible under higher resolution and scattered throughout the cytoplasm. Scale bar = 1 μm.



FIGS. 10–12. Showing small regular particle (SRP). 10) As for Figure 8 but showing the SRP (arrows) under higher resolution in the vicinity of a crystalloid (cr). Scale bar = 0.1 μm. 11) SRP (arrows), under high resolution, lining the esophagus of *E. heptapapillatus*. Scale bar = 50 nm. 12) TEM micrograph of a cross section of *E. heptapapillatus*. Showing the distribution of SRP throughout the cytoplasm of the somatic muscle and their absence from the cuticle (c). Scale bar = 1 μm.

croscope (SEM) operated at 20 kV. The best results for this type of analysis were obtained using whole crystalloids shed from the ends of fragments of cut nematodes that had been fixed in aqueous 4% formaldehyde and then not treated with any other chemical. Thin sections of these nematodes were also examined in a Jeol 2000-FX TEM equipped with a Tracor Northern EDX.

Results

Living specimens of *E. heptapapillatus* contained clearly visible crystalloid bodies (Figs. 1, 2) that exhibited autofluorescence. Histochemical tests showed that the bodies contained carbohydrate (PAS positive), lipid (osmium tetroxide positive), and some protein (weakly BBG positive). These crystalloids occurred in the pseudocoelom, rather than in muscle or intestinal cells, and they moved up and down in surges in this cavity as the nematode moved. They did not appear to impede normal physiological processes such as movement or egg laying. Their distribution is perhaps best illustrated in 1-μm cross sections of the resin-embedded nematode viewed under the SEM (Fig. 3), which show the crystalloids lying in the pseudocoelom between the somatic musculature and the esophagus. Analysis for elements using EDX attached to the SEM was done on these cross sections (Fig. 4). The nematode from which these sections were cut had been routinely stained with osmium tetroxide to increase contrast prior to dehydration and embedding. The presence of this and various elements in the embedding medium tended to complicate the spectral analyses. This was partly overcome by taking an EDX spectrum of the embedding medium alone (Fig. 4D) in the area indicated in Figure 3 and subtracting this background reading from those taken of one of several crystalloids (Fig. 4A), somatic muscle (Fig. 4B), and the esophagus (Fig. 4C) from the sites indicated in Figure 3. It can be seen from these EDX spectra that the crystalloids contain sulfur (Fig. 4A), which is not found in other, similarly-treated parts of the nematode (Fig. 4B, C). Similarly treated 5-μm-thick longitudinal-tangential sections of nematodes in which crystalloids extruded from their cut ends (Fig. 5) were examined under the SEM. An X-ray distribution map of part of the area shown in Figure 5 for the elements shown in Figure 4A–C shows that the mass of crystalloids contains sulfur and either osmium or phosphorus, or both, and does not contain chlorine. The presence of the osmium M peak masks the phosphorus K peak in material that has been fixed in osmium. To overcome this we analysed whole crystals extruded from the cut ends of nematodes that had been fixed only in formaldehyde and not treated with any other chemical (Fig. 6). The EDX spectra of these crystals (Fig. 9A) clearly show that phosphorus is present in addition to sulfur.

These crystalloids vary considerably in size up to a maximum length of ca. 10 μm and are oval in shape (Figs. 7, 8). They occurred in *E. heptapapillatus* in all samples collected at regular monthly intervals over a 12-month period and were found in most specimens examined. Thus of 204 specimens examined from the Mundoo site, only six (3%) appeared to be free of crystalloids, and of 95 specimens examined from the bottom of Lake Alexandrina, only three (3%) appeared to be free of crystalloids. They occur in both larvae and adults and appear to be associated with small regular particles (SRP), ca. 20 nm d (Figs. 10, 11). The number of these SRP per nematode varies. Most of the 40-crystalloid-containing nematodes examined under the TEM at high resolution contained them, but they were not detected in nematodes that did not have crystalloids, including those obtained from South Africa which, although having been in glycerol, still gave a good TEM image when sectioned after rehydration, staining, dehydration, and embedding.

In those specimens containing large numbers of SRP, the particles occurred in all tissues except the crystalloids (Fig. 10) and the cuticle (Fig. 12). They may be seen lining the esophageal lumen (Fig. 11) and

may be ingested. These structures appear too small and uniform in shape to be caused by staining artifacts; under high resolution (Fig. 11) they resemble some icosahedral viruses in size and shape, but we have no other evidence to support the contention that they may be viruses. Analyses of these SRP for both iron and manganese using EDX attached to the TEM were negative, and it seems unlikely that these structures are metallic deposits of any type.

Discussion

The occurrence of crystalloid bodies in a number of genera of aquatic free-living nematodes has been thoroughly reviewed (4,8). In studies with *Tobrilus gracilis,* collected from the river Elbe and from a lake near Bremerhaven, Nuss (8) found that during the summer months (May–October) up to 95% of these nematodes contained crystalloids, whereas during the winter months (November–April) only 50% of this population contained crystalloids. He reported that these crystalloid structures, which were mostly found in the somatic muscle and not in the gut or pseudocoelom, were "part of a detoxification system for sulfide ions." Nicholas et al. (7) concluded that inclusions in the species they examined are also associated with detoxification of sulfide ions, but by the deposition of insoluble metal sulfides rather than the oxidation of H_2S to elemental sulfur. The crystalloids in *E. heptapapillatus* do not appear to have this function because these nematodes were found to occur more commonly in the top sandy 5 cm of soil rather than in the darker, more sulfur-rich 5–10-cm-deep layer.

Nematodes kept in aquaria for periods of 2 months, during which they grew and laid eggs, appeared to be free of crystalloids. Loss of crystalloids during cultivation has been observed also in other tobrilids (Nicholas, pers. comm.). In view of these observations, we conclude that these sulfur and lipid-containing crystalloid structures may be vehicles for food storage for survival during periods of food scarcity, such as may occur during in vitro culture, environmental pollution episodes, or during the winter months when food sources may decrease. This might explain the observations that the crystalloids become depleted during the winter months (8) and that the crystalloid inclusions in *Haemonchus contortus* may be "cleared away by the worms" (3) and so do not appear to represent a pathological state, as was formerly thought (2). It should be pointed out, however, that these crystalloid structures bear no resemblance to the lipid droplets that act as food reserves in many tylenchid nematodes. These lipid droplets are consumed rapidly when these nematodes are placed in shallow distilled water at their temperature optima (11). In contrast, the crystalloids of *E. heptapapillatus* remain intact under the same conditions and show no changes.

Accumulation of iron and manganese has been observed in the Australian fresh water mussel *Velesunio ambiguus* in the River Murray, whose waters are unusually high in these elements (5). It was thought that the crystalloids in *E. heptapapillatus* might act as temporary storage bodies for heavy metal complexes. However, the absence of iron and manganese in the crystalloids of this nematode does not support the concept that they might be stored excretory products resulting from the ingestion of these elements.

It is interesting to note, in passing, that good TEM images were obtained from glycerol-mounted material from South Africa. Therefore, museum specimens can be valuable sources of information. In our studies, neither crystalloids nor SRP were observed in some specimens, including the glycerol-mounted material from South Africa. The presence of the 20 nm SRP which appear to be associated with these crystalloids is difficult to explain other than that they indicate a pathological state, perhaps induced by environmental pollution. It is interesting to note in this regard that where the SRP occur in large numbers, the tissues around them appear to be breaking down (Figs. 7, 8, 12). We hope to pursue these studies.

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