Flux of cadmium through the marine nematode *Enoplus brevis* Bastian, 1865

Richard HOWELL and Lawrence SMITH

Department of Zoology, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, England.

SUMMARY

Pulse-labelling with ^{109}Cd was used to study the movement of the metal through the marine nematode *Enoplus brevis*. When exposed to filtered seawater containing ^{109}Cd as cadmium chloride, the metal first appeared in association with the cuticle. Polyacrylamide gel electrophoresis demonstrated that the metal was being bound in the cuticle by a protein with a molecular weight (M.W.) of 450,000 Daltons. ^{109}Cd was subsequently detected in the hypodermis and muscle layers where it was associated with a protein of M.W. 28,000 Daltons, and finally it appeared to accumulate in the gut, associated with a third protein of M.W. 200,000 Daltons. When exposed to ^{109}Cd-labelled sediment, there was little uptake by the cuticle but a marked uptake and loss over several days by the gut, where the metal appeared to be bound by the same protein of M.W. 200,000 Daltons.

RÉSUMÉ

Le flux du cadmium à travers le nématode marin Enoplus brevis *Bastian, 1865.*

Le mouvement du métal dans le nématode marin *Enoplus brevis* a été étudié par la méthode dite du « pulse-labelling ». Après immersion du nématode dans de l'eau de mer filtrée contenant du ^{109}Cd, ce métal apparaît d'abord être associé à la cuticule. L'électrophorèse sur gel polyacrylamide révèle que le métal est lié à la cuticule par l'intermédiaire d'une protéine dont le poids moléculaire (P.M.) est de 450 000 Daltons. Ensuite, le ^{109}Cd est décélé dans l'hypoderme et les couches musculaires, lié à une seconde protéine de P.M. 28 000 Daltons. Enfin, il semble s'accumuler dans l'intestin où il est lié à une troisième protéine de P.M. 200 000 Daltons. En revanche, lorsque le nématode est mis au contact de sédiments marqués par le ^{109}Cd il n'y a pas d'absorption par la cuticule tandis que l'absorption par l'intestin, beaucoup plus forte, est suivie d'une perte après plusieurs jours; dans ce dernier cas, le métal est lié à la même protéine de P.M. 200 000 Daltons.

The importance of cadmium as a pollutant of the marine environment has been recognised for some time. It is a highly toxic metal and is introduced to the sea as a by-product of several industrial processes. It is accumulative and may be concentrated by organisms to levels well in excess of those in the environment (Douglas-Wilson, 1972; Eisler, 1981; Wright, 1978). In addition, being strongly electronegative it is readily adsorbed by organic material in marine sediments where it becomes available to the benthos (Luoma & Jenne, 1975; Phelps, 1979).

Nematodes form a significant, sometimes dominant, part of the meiobenthos of marine sediments (McIntyre, 1969; Platt & Warwick, 1980) and yet the group has been almost totally ignored in pollution studies. Recent work (Howell, 1982 a, 1982 b, 1983, 1984) has indicated that some species of nematode are very suitable for studies concerning the physiology of accumulation of heavy metals; this work has been extended to investigate the flux of cadmium through nematodes. In addition it has been demonstrated by Howell and Smith (1983) that metal-binding proteins can be isolated using polyacrylamide electrophoresis from body extracts of the nematodes studied. In this communication, results of further work are presented in which these metal-binding proteins are shown to occur in different tissues of the body.

Materials and methods

All glassware used in the following experiments was acid-washed and thoroughly rinsed in deionised water before use. Glassware used to contain radioactive material was treated with the silicone-coating reagent Siliclad. All chemicals used were of analytical grade.

Details of collection, exposure and analysis of the nematodes are given below.

COLLECTION

The marine nematode *Enoplus brevis* Bastian, 1865 was collected by sieving sediment from Budle Bay, Northumberland, an unpolluted site (R. Howell, unpubl. data) and removing the nematodes from the sieve by a mounted needle and hand lens. Nematodes were then narcotised in a 2 % solution of propylene phenoxitol in seawater (Ellenby & Smith, 1964), subjected to mild ultrasonication to remove gut contents (Howell, 1982 b) and then rinsed several times in clean seawater.

EXPOSURE TO ^{109}CD-LABELLED SEAWATER

Approximately 100 nematodes were exposed for 12 hours to seawater containing 3 mg l^{-1} cadmium chloride and 100 µCi 50 ml^{-1} ^{109}Cd giving the solution a high level of activity (1.64 × 10^5 cpm µM Cd^{-1}). ^{109}Cd was obtained from Amersham International plc, England. After this period the nematodes were rinsed in clean seawater and placed into unlabelled seawater containing 3 mg l^{-1} cadmium chloride for a further period of 11.5 days. Animals were exposed in covered Petri dishes, containing a sterile, artificial substrate of Ballatini glass beads (Anderson & Coleman, 1977), in a constant temperature room at 10°. Solutions were not aerated but were changed every 24 hours.

Groups of twenty animals were removed at intervals of 0, 1, 3, 6 and 12 days from the start of the experiment and the tissues analysed for total cadmium and ^{109}Cd as described later.

EXPOSURE TO ^{109}CD-LABELLED SEDIMENT

Sediment from the Blyth estuary, Northumberland, known from previous work to be significantly contaminated by cadmium (R. Howell, unpubl. data) was collected and sieved to remove nematodes and other members of the meiofauna. The filtrate and washings were collected and after mixing with the sediment retained by the sieve, the mixture was evaporated down at room temperature until a residue with the approximate consistency of the original sediment was obtained. A sample of this reconstitued sediment was placed into ^{109}Cd-labelled seawater containing 100 µCi 100 ml^{-1} for 1 hour and then the mixture was again evaporated down at room temperature until the sediment resembled natural Blyth estuary sediment.

Approximately 100 nematodes were exposed to this labelled sediment in Petri dishes maintained in a constant temperature room at 10° as before. After 12 hours exposure the animals were collected, rinsed in clean seawater and placed into unlabelled Blyth sediment from which the meiofauna had been removed by sieving as described earlier. The animals were exposed to this sediment for a further 11.5 days.

Samples of twenty animals were removed at time intervals of 1, 3, 6 and 12 days from the start of the experiment and treated as follows.

DISSECTION AND ANALYSIS OF TISSUES

Groups of nematodes taken from the exposure media (seawater or sediment) were rinsed in clean seawater and narcotised to the death point using a 2 % solution of propylene phenoxitol in seawater. They were then subjected to mild ultrasonication to remove gut contents after which individuals were dissected into six regions as follows. The pharynx, up to the oesophago-intestinal junction, and the tail, up to the anal aperture, were removed first. The remainder was carefully manipulated to produce a gut tube, a cuticular tube, the eggs, and « other tissues ». The latter included the hypodermis and muscle layers and the reproductive organs which could not be successfully separated from one another.

Tissues from the twenty animals in each group were pooled into two groups of ten, dried on ashless filter paper and weighed. The pooled tissues were then homogenised in 0.1 ml of 0.3 M sucrose at 0° and the homogenate subjected to ultrasonication for 10 minutes at 0° as described by Howell and Smith (1983). 5 µl aliquots of the tissue extracts were used to determine the total cadmium concentration by flameless atomic absorption spectrophotometry using a Varian Techtron atomic absorption spectrophotometer fitted with a carbon rod atomiser. A 50 µl aliquot was taken from each tissue extract and added to 10 ml of NE 262 scintillant and then analysed for ^{109}Cd activity using a Beckman LS 100 C liquid scintillation counter, set to a counting efficiency of 2 %.

DETERMINATION OF THE MOLECULAR WEIGHTS OF THE OBSERVED CADMIUM-BINDING PROTEINS

Analysis of total cadmium and ^{109}Cd activity in the different tissues indicated that the eggs, tail and pharynx were of minor importance to the flux of cadmium through the nematodes during the experiment. It was also noted that peaks of ^{109}Cd activity in the cuticle, gut and " other tissues " occured at days 1 and 3 after the start of the experiment. It was therefore decided to confine the final part of the experiment to those tissues at those times to maximise the use of the available animals.

Approximately 100 nematodes were exposed to ^{109}Cd-labelled seawater for 12 hours and then to unlabelled seawater containing 3 mg l^{-1} cadmium as cadmium chloride for a further period of 2.5 days. Similarly 100 nematodes were exposed to labelled, then unlabelled Blyth sediment. Groups of 50 animals were taken from the exposure media 1 and 3 days after ths start of the experiment.

The gut contents were removed as described previously, the nematodes were dissected into the six regions and protein extracts were made of the different tissues as described by Howell and Smith (1983).

Polyacrylamide gel electrophoresis was carried out according to the method of Hendrick and Smith (1968). Replicate 25 µl aliquots of the protein extracts from the tissues were mixed with 5 µl of Bromophenol Blue tracking dye and run at the same time in gels with 4 %, 7.5 % and 12 % cross-linking, using a basic buffer. There were two gels at each level of cross-linking, one of which was stained for protein and the other used to detect ^{109}Cd activity. Five standard protein markers were run under the same conditions and all gels were stained for protein using the following procedure. Gels were first fixed in

an isopropanol/acetic acid/deionised water mixture (Anon., 1974). Proteins were then stained using Coomassie Blue (Swank & Munkres, 1970). Destaining was by a Shandon Southern transverse destainer. Gels were quantified using a Joyce-Lobel densitometer.

^{109}Cd activity was estimated by liquid scintillation counting after preparation according to Fillingame (1975).

A mixture of proteins run in basic buffer does not separate according to molecular weight as occurs in sodium dodecyl sulphate (S.D.S.) electrophoresis. However if the same protein mixture is run in several gels of different percentage cross-linking the relative mobility (Rm) of a particular protein in the different gels correlates with its molecular weight (Hendrick & Smith, 1968). The relative mobility of each standard protein in each gel was expressed as 100 × log (Rm × 100). These values were plotted against the gel percentage cross-linkage as shown in Figure 1. The slopes of the lines which were calculated are proportional to the molecular weights of the proteins. Thus a plot of M.W. against slope produces a straight line (Fig. 2) allowing the molecular weights of the unknown proteins to be determined.

Results

The results of the analyses of total cadmium and ^{109}Cd activity in nematodes exposed to cadmium in seawater are shown in Figure 3. Figure 4 shows the results in

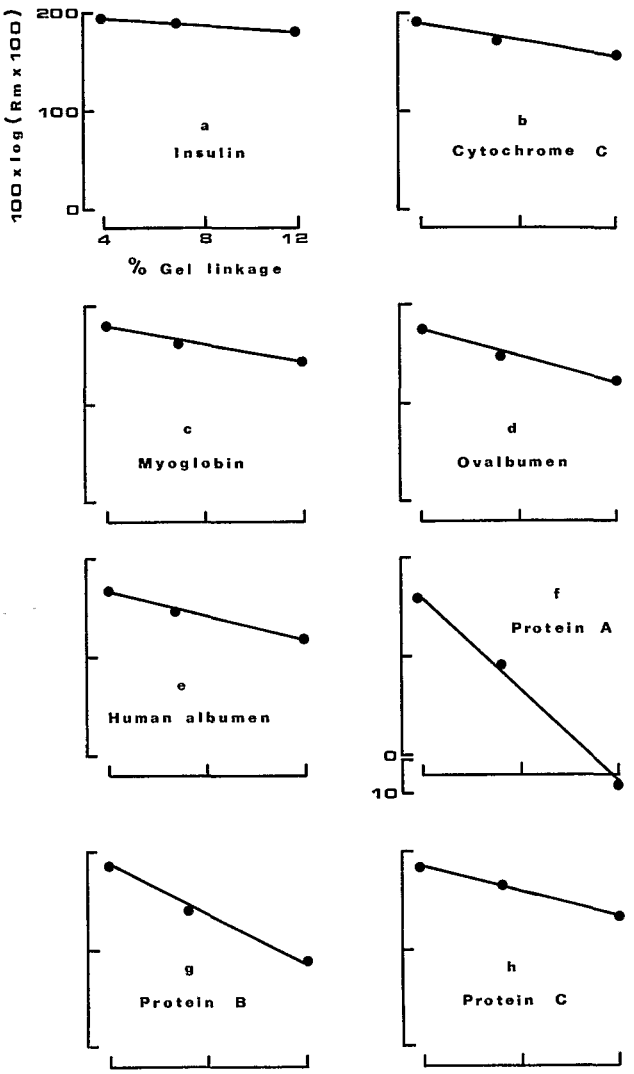

Fig. 1. Gel cross-linkage against the value 100 × log (Rm × 100) of the standard proteins (a-e) and the unknown cadmium-binding proteins (f-h) following electrophoresis under the conditions of Hendrick & Smith (1868). Axes labelled as for insulin in each case.

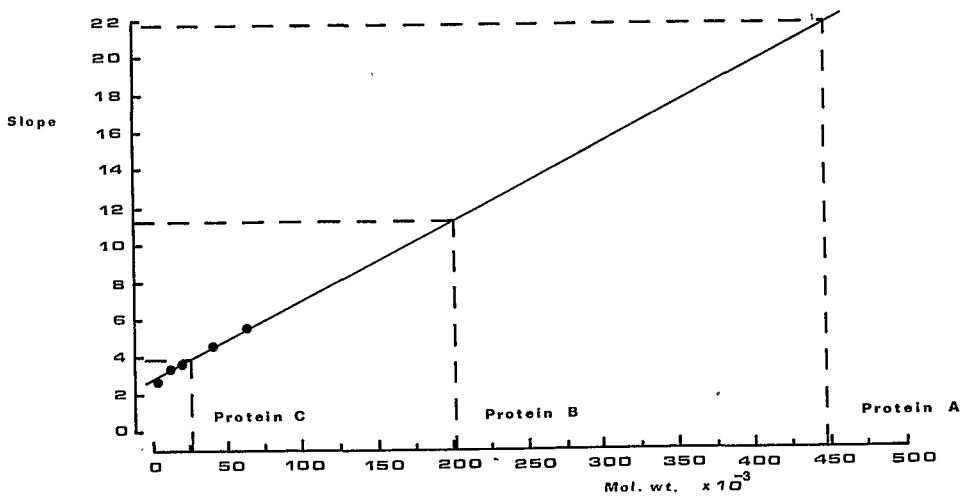

Fig. 2. Molecular weight against slope of the standard proteins, showing the positions of the unknown proteins following electrophoresis under the conditions of Hendrick and Smith (1968).

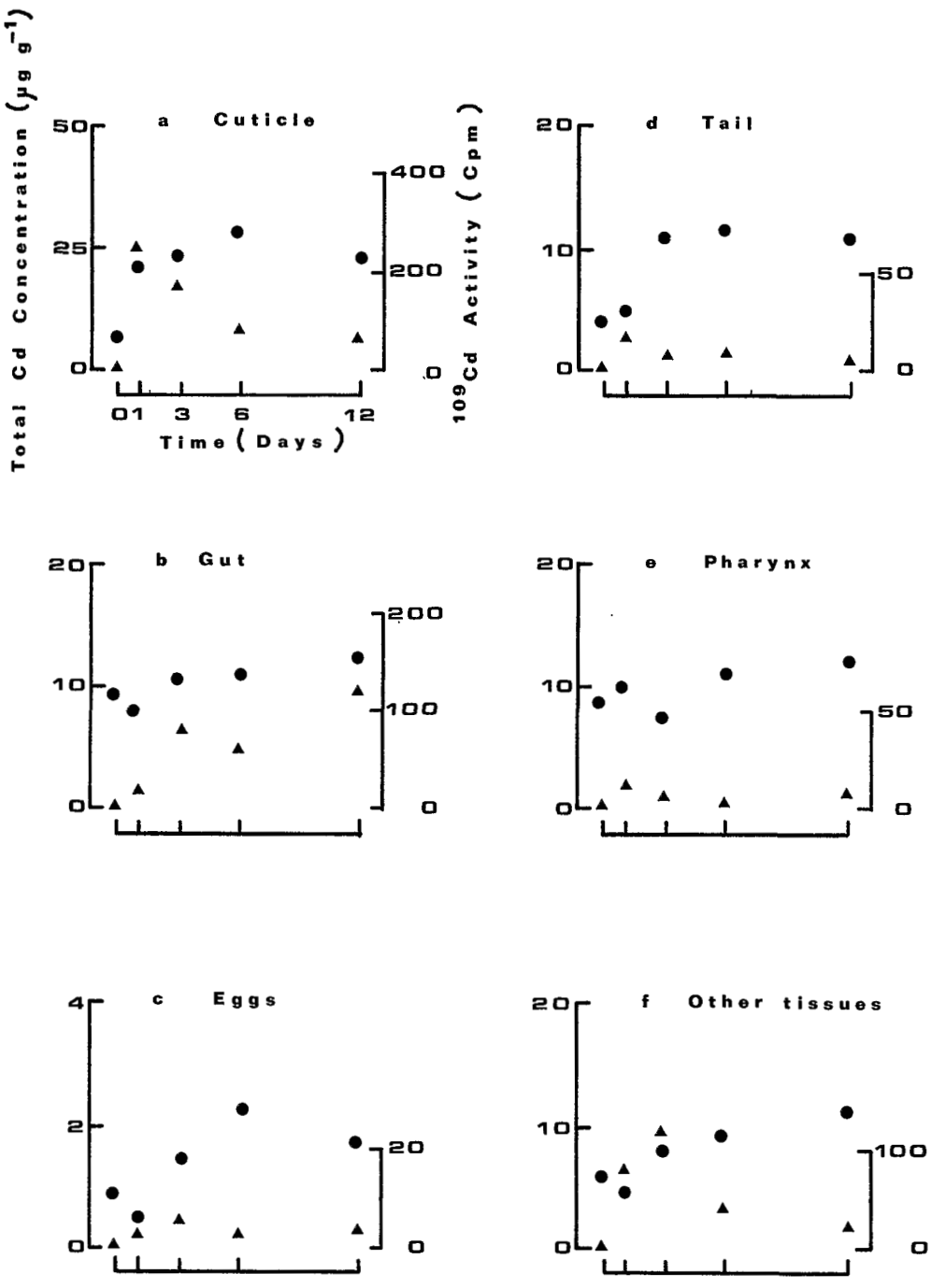

Fig. 3. Graphs of total cadmium concentration (black circle) and ^{109}Cd activity (black triangle) in tissues of *E. brevis* following exposure to cadmium-labelled seawater. Each point represents the mean of two pooled groups of ten animals. Axes labelled as for the cuticle in each case.

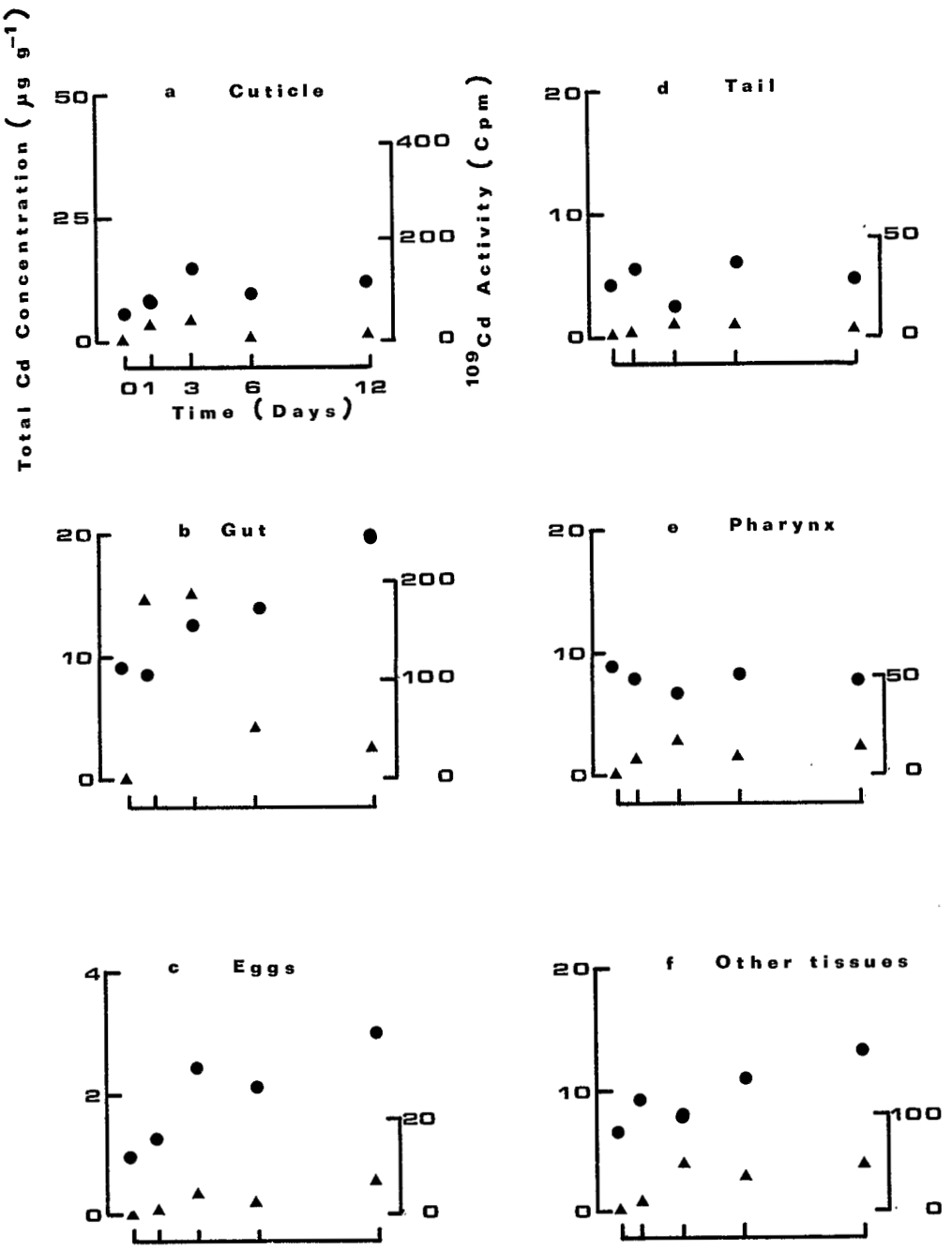

Fig. 4. Graphs of total cadmium concentration (black circle) and ^{109}Cd activity (black triangle) in tissues of *E. brevis* following exposure to cadmium-labelled sediment. Each point represents the mean of two pooled groups of ten animals. Axes labelled as for the cuticle in each case.

animals exposed to cadmium in sediment. In both cases it appears that the eggs, tail and pharynx do not play an important role in the movement of cadmium through the nematodes.

Detection of ^{109}Cd activity in the different tissues demonstrated the presence of three cadmium-binding proteins of different molecular weights. These were estimated to be (A) 450,000 Daltons (B) 200,000 Daltons (C) 28,000 daltons. Proteins A and C have been observed previously in whole animal extracts (Howell & Smith, 1983) but protein B has not been demonstrated before, Protein A was present in extracts from the cuticle, and in the tail and pharynx (presumably by virtue of their cuticular component). Protein B was isolated in the gut and pharynx and protein C in the tail and « other tissues ».

Discussion

It appears from Figures 3 and 4 that most of the cadmium flux occured through the cuticle, gut and " other tissues ". However the temporal relationship between the ^{109}Cd activity peaks in these tissues were different depending on the exposure medium used (seawater or sediment). In the eggs, tail and pharynx there was little change in the ^{109}Cd activity during the period of the experiment.

In the cuticles of nematodes exposed to cadmium in seawater there was an immediate and continuous uptake of metal up to Day 6 when a plateau level was established. This type of response has been observed several times during studies on metal uptake by organims (Wright, 1978). A ^{109}Cd peak occured after 1 day of exposure but this subsequently declined in the cuticle despite the continued uptake of cadmium from seawater solution (Fig. 3). The loss of ^{109}Cd activity by the cuticle was not complete : some remained and by the end of the experiment (Day 12) the activity was approximately 20 % of that at Day 1. Since the total cadmium concentration of the cuticle increased up to Day 6 it seems unlikely that the loss of ^{109}Cd activity from the cuticle was due entirely to deabsorption from the cuticular surface.

The gut showed a relatively lower level of uptake but at a significant rate throughout the period of exposure. There appeared to be a short time lag before the concentration in the gut started to rise. ^{109}Cd activity showed a peak on Day 3 after which the level remained high during the remainder of the experiment. Since it has been shown that *E. brevis* does not imbibe significant amounts of seawater in the absence of sediment (R. Howell, unpubl. data), it seems likely that any uptake of cadmium by the gut must be due to passage through, or translocation from, the cuticle. This hypothesis would then explain the temporal relationship between ^{109}Cd activity maxima observed in the cuticle and the gut.

Similarly, the incresase and decrease in ^{109}Cd activity observed in the " other tissues " is then explained since these tissues lie between, and contiguous with, the cuticle and the gut. Movement of the cadmium, probably in bound form, from the cuticle through the " other tissues " to the gut may be mediated by the wandering phagocytic cells known as stellate cells and which are found usually in the pseudocoelom (Chitwood & Chitwood, 1974).

Different patterns of cadmium uptake were observed in the tissues of nematodes exposed to labelled sediment although as in the case of seawater exposed animals, most of the cadmium flux occured through the gut and " other tissues ".

There was relatively little uptake by the cuticle. This was probably due to the capacity of organic constituents of Blyth sediment to bind the strongly electronegative cadmium ions, thus reducing the concentration of dissolved cadmium and the amount available for surface adsorption (Phelps, 1979). Once sedimentary material has been taken into the gut, changes in the physicochemical environment may lead to the release of cadmium which then becomes available for uptake by the gut tissues. Thus there was a rapid increase in ^{109}Cd activity in the gut tissues of nematodes exposed to labelled sediment. However, much of this activity was lost by the gut by the end of the experiment (Fig. 4). There was no commensurate rise in ^{109}Cd activity in the cuticle or the " other tissues " and it is suggested that the loss of activity from the gut was due to the removal of cadmium from the nematode via the gut. The increase in total cadmium concentration measured in the gut was probably due to translocation of the metal from different tissues of the body leading to saturation of the excretory mechanism and subsequent storage in the gut wall (see later). The removal of material in this way may only occur when sedimentary material is passing through the gut because in the absence of this movement, the high internal hydrostatic pressure prevents normal defecation. This also suggests that part of the gut is concerned with absorption and part with the removal or " excretion " of material probably by exocytosis (Crofton, 1968; Jennings & Colam, 1970). In the case of nematodes exposed to cadmium in seawater, the loss of material from the gut lumen does not occur and the gut tissues then act as a " kidney of accumulation " as suggested by Jennings & Colam (1970), resulting in the rise in ^{109}Cd activity observed in Figure 3 b.

There was a small increase in ^{109}Cd activity in the " other tissues " from Day 3 of the experiment, reaching a plateau level by Day 6. It is possible that this represents « spill-over » from the gut tissues, suggesting that the storage or excretory capacity of these tissues was being exceeded.

Determination of the molecular weights of the cadmium-binding proteins indicated the presence of an additional protein (Protein B) not previously demonstrated in whole animal extracts (Howell & Smith, 1983). It

was present in the pharynx and the " other tissues " and its incidence at these sites may offer a clue as to its identity. The pharynx contains a structure known as the ventral gland which secretes an Alcian Blue positive mucus (Howell, 1982 a). This has been demonstrated to be highly effective at binding heavy metals and may play a role in heavy metal metabolism by the nematodes (Howell, 1982 a). It is also known that buccal glands and digestive glands in the pharynx and foregut secrete mucus and that this passes through the gut tube, to form a lining to the gut as described by T. King (unpubl. observ.). This lining could function to protect the gut wall from mechanical abrasion by imbibed sediment or as suggested by T. King (pers. comm.) it could function as an ion exchange mechanism resulting in the binding of cadmium ions by the gut tissues. Alternatively the gut wall could be binding cadmium directly, possibly utilising protein C which may be acting as a storage system for detoxifying the metal. Cadmium, bound by protein C, could be retained in a detoxified state until it was removed from the body by exocytosis into the gut lumen. This proposed mechanism resembles the system described in the hepatopancreas of crustaceans and in the kidney of molluscs (Brown, 1978; George *et al.*, 1978).

The isolation of protein C from the cuticle provides further evidence to support the suggestion of Howell & Smith (1983) that it is a collagen-type component of the cuticle.

Clarification of the form of metabolised cadmium immediately prior to its removal from the nematode could be achieved using electron probe microanalysis and work of this type is now in progress. The results of this study will be presented in a further communication.

References

Anderson, R. V. & Coleman, D. C. (1977). The use of glass microbeads in ecological experiments with bacteriographic nematodes, *J. Nematol.*, 9 : 319-322.

Anon. (1974). *BioRad Ltd Technical Bulletin*, 1024.

Brown, B. E. (1978). Lead detoxification by a copper-tolerant isopod. *Nature*, 276 : 388-390.

Chitwood, B. G. & Chitwood, M. B. (1974). *Introduction to Nematology.* Baltimore, MD, U.S.A., Univ. Park Press, 334 p.

Crofton, H. D. (1968). The alimentary canal. In : *Nematodes*, London, Hutchinson Univ. Library : 69-81.

Douglas-wilson, I. (1972). Cadmium pollution and Itai-Itai disease. *Lancet*, 1 : 382-383.

Eisler, R. (1981). *Trace metal concentrations in marine organisms.* London, Pergammon Press, 687 p.

Ellenby, C. & Smith, L. (1964). A narcotic and an immersion medium for living nematodes with some observations on the refractive index of the cuticle. *Nematologica*, 10 : 342-343.

Fillingame, R. H. (1975). Identification of the dicyclohexylcarbodiimide-reactive protein component of the adenosine 5 -triphosphate energy-inducing system of *Escherichia coli. J. Bacteriol.*, 124 : 870-883.

George, S. G., Pirie, B. J. S., Cheyne, A. R. Coombs, T. L. & Grant, P. T. (1978). Detoxification of metals by marine bivalves : An ultrastructural study of the compartmentation of cooper and zinc in the oyster *Ostrea edulis. Mar. Biol.*, 45 : 147-156.

Hendrick, J. L. & Smith, A. J. (1968). Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Arch. Biochem. Biophys.*, 126 : 155-164.

Howell, R. (1982 a). The secretion of mucus by marine nematodes (*Enoplus* spp.) : A possible mechanism influencing the uptake and loss of heavy metal pollutants. *Nematologica*, 28 : 110-114.

Howell, R. (1982 b). Levels of heavy metal pollutants in two species of marine nematode. *Mar. Pollut. Bull.*, 13 : 396-398.

Howell, R. (1983). Heavy metals in marine nematodes : Uptake, tissue distribution and loss of copper and zinc. *Mar. Pollut. Bull.*, 14 : 263-268.

Howell, R. (1984). Acute toxicity of heavy metals to two species of marine nematodes. *Marine Environ. Res.*, 11 : 153-161.

Howell, R. & Smith, L. (1983). Binding of heavy metals by the marine nematode *Enoplus brevis* Bastian, 1865. *Nematologica*, 29 : 39-48.

Jennings, J. B. & Colam, J. B. (1970). Gut structure, digestive physiology and food storage in *Pontonema vulgaris* (Nematoda : Enoplida). *J. Zool., Lond.*, 161 : 211-221.

Luoma, S. N. & Jenne, E. A. (1975). Factors affecting the availability of sediment-bound cadmium to the estuarine, deposit feeding clam, *Macoma balthica.* In : Cushing Jr., C. (Ed) *Radioecology and energy resources. Symposium on radioecology.* Oregon State Univ. Press : 283-290.

McIntyre, A. D. (1969). Ecology of marine meiobenthos. *Biol. Rev.*, 44 : 245-290.

Phelps, H. L. (1979). Cadmium sorption in estuarine mud-type sediment and the accumulation of cadmium in the soft-shell clam, *Mya arenaria. Estuaries*, 2 : 40-44.

Platt, H. M. & Warwick, R. M. (1980). The significance of free-living nematodes to the littoral ecosystem. In : Price, J. H., Farnham, W. F. & Irvine, D. E. G. (Eds) *The shore environment.* London, Academic Press : 729-759.

Swank, R. T. & Munkres, K. D. (1970). Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulphate. *Analyt. Biochem.*, 39 : 462-477.

Wright, D.A. (1978). Heavy metal accumulation by aquatic invertebrates. *Appl. Biol.*, 3, 331-394.

Accepté pour publication le 19 novembre 1984.