SLUGS AS TARGET OR NON-TARGET ORGANISMS FOR ENVIRONMENTAL CHEMICALS

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ABSTRACT

The ability of slugs (Deroceras reticulatum) to survive exposure to various chemical stressors such as molluscicides (carbamates, metaldehyde, iron-chelates) or other environmental pollutants (heavy metals, pesticides) was investigated using x-ray analyses, autoradiography, filter electron rnicroscopy, enzyme histochemistry, enzymatics, electron microscopy, and stress protein analyses. Uptake pathways, target sites, modes of action and general responses in the animnals were studied, and the results (including aspects of resorption and metabolism of the toxins as well as their cellular and biochemical effects) interpreted in terms of both molluscicidal efficiency and of the consequences to slugs' ecological role as soil decomposers.

INTRODUCTION

Slugs, particularly Deroceras reticulatum, are important pests in agriculture and horticulture in many parts of the world (Barker, 1989, Beer, 1989, Byers & Calvin, 1994, Ferguson, 1994). As with many other pest species, their pest status arose mainly from Man's activities favouring high population densities of slugs, for example by creating monocultures of favoured food, by cultivation methods which provide refuges from desiccation and predation, and by providing glasshouses in which slugs can escape from both harsh winters and dry summers. In nature however, slugs, like earthworms, are ecologically important decomposers of plant litter in woodland and other soils (e.g. Swift *et al.*, 1979). Slugs are therefore of interest not only to agriculturists and the pesticide industry but also to ecotoxicologists as monitor species for environmental pollution. Both disciplines are interested in similar endpoints of toxicity: 1) Mortality, as the best (for pest control) or the worst case (for ecotoxicologists) of toxic impact; 2) Cell damage as evidence of molluscicidal effects or as an early warning marker for sublethal environmental pollution; and 3) The organisms' defense mechanisms, as barriers to be overcome in molluscicide development or as a strategy for survivial in a contaminated environment.
This paper investigates the toxicology of slugs in both situations: 1) Slugs as target organisms for molluscicides (methiocarb, cloethocarb, metaldehyde or iron chelates), and 2) Slugs as non-target organisms exposed to environmental pollutants (heavy metals, γ-HCH (lindane) or pentachlorophenol (PCP). Responses at the organ, cell and biochemical levels and their energetic implications are discussed.

MATERIALS AND METHODS

Test animals

Adult Deroceras reticulatum, either laboratory-reared and between four and six months old or field collected and between 400 and 600 mg weight, were used in all the experiments.

Application of substances

All the molluscicides were administered orally. The compounds used were 1) Commercially available slug pellets [Mesurol - 4% of 4-(methylthio)-3,5-xylyl-methyl carbamate and *Spiess Urania 2000* - 4% metaldehyde]; 2) Dried wheat germ agar containing either 2% or 0.1% cloethocarb [phenol-2-(2-chloro-1-methoxyethoxy) methylcarbamate]; 3) Agarose gel containing either 23 µg or 63 µg metaldehyde per animal directly injected into the buccal cavity; and 4) Whole wheat flour (5mg) containing either 30 µg or 500 µg per animal of either iron butan [tris(1-oxo-1,2-diazabutan-2 oxido)Fe(III)] or iron octan [tris(1-oxo-1,2- diazaoctan-2-oxido)Fe(III)] (Clark *et al.*, 1995).

Heavy metals at various concentrations (59, 358 or 751 µg Cd/g dry wt; 405, 2456 or 3751 µg Pb/g dry wt; 1874, 9305 or 14915 µg Zn/g dry wt) or γ-HCH (58, 116 or 231 ng/g dry wt) or PCP (29, 87 or 867 ng/g dry wt) were also given orally by feeding the animals lettuce leaves soaked with aqueous solutions of each toxin. The slugs fed with heavy metals were also exposed to 1.4, 5.8 or 14.1 µg Cd/g dry wt, 103.1, 164.6 or 292.3 µg Pb/g dry wt or 136.4, 86.9, or 490.9 µg Zn/g dry wt in the substrate.

For all tests, control animals were kept on uncontaminated substrate and fed uncontaminated food.

Tracing methods

X-ray analysis: Slugs were fed a standard food (Standen, 1951) containing 20% ThO_2 and 2% cloethocarb and x-rayed at fixed intervals after feeding began (method as in Triebskorn & Florschütz, 1993).

Auioradiography: Slugs were fed either ^{14}C-labeled cloethocarb (1 µCi) or metaldehyde (3 µCi) and dissected at fixed intervals after feeding began (method as in Triebskorn *et al.*, 1990).

Filter electron microscopy: Slugs were fed with low (30 µg or 0.6%) or high (500 µg or 10%) concentrations of both iron octan and iron butan, and dissected and fixed 16 hours after feeding began. Samples of hepatopancreas and skin were fixed in 2% glutardialdehyde in 0.01 M cacodylate buffer (pH 7.4) (2h at 4°C), then 1% aqueous OSO_4 (2h at 4°C), then embedded in Spurr resin. Ultrathin sections were analyzed for iron according to the method described by Triebskorn *et al.* (in press). Similarly prepared sections of hepatopancreas of

metal-treated slugs were analyzed for zinc according to the method described by Triebskorn & Köhler (in press).

AAS (atomic absorption spectroscopy) measurements: The metal content of slugs was determined according to the method described by Köhler *et al.* (1995), and Triebskorn & Köhler (in press).

Structural investigations

For light and electron microscopy slugs were dissected at fixed intervals after feeding began and fixed in 2% glutardialdehyde in 0.01 M cacodylate buffer (pH 7.4). The samples were either routinely processed for electron microscopy (e.g. Triebskorn, 1989; Triebskorn & Künast, 1990) or for paraffin histology (e.g. Triebskorn, 1995).

Enzvme histochemical tests and enzymatics

For enzyme histochemical tests tissues were either frozen in liquid nitrogen or fixed for 1 h in 2% glutardialdehyde. Fixed material was embedded, without dehydration, in *HistoResin*. Non-specific esterases (UE), alkaline phosphatase (AP), acid phosphatase (AcP), Ca-ATPase (ATP) and neotetrazoliumreductase (NTR) were investigated according to the methods described by Triebskorn (1991, 1995). In parallel, enzyme activities of AP, AcP and arylhydrocarbonhydroxylase (AHH) were detemnined photometrically according to Triebskorn (1991).

Stress protein analyses

In animals exposed to heavy metals, the stress protein (hsp70) was analyzed by a standardized Western blotting technique and subsequent image analysis according to Köhler *et al.* (1992, 1994) andZanger *et al.* (1996).

RESULTS

Contact, absorotion, and storage

Slugs encounter toxic materials either by contact or during feeding. If they crawl over surfaces recently sprayed with pesticides, on toxic slug baits or in heavily polluted substrates the skin is the first point of contact. However, the primary target for toxic baits and the point of first contact for pollutants ingested with contaminated food is the digestive tract. For stomach poisons such as molluscicidal baits the foregut and crop can be the main site of absorption. X-ray studies of animals fed with 2% cloethocarb showed that the transport of the food pulp along this part of the gut was inhibited, and the molluscicide retained there for at least 15h. Direct cellular effects on the foregut and crop and the enhanced opportunity for toxins to be absorbed there could therefore both be important factors in the toxicity of such compounds.

Autoradiography of animals fed with radiolabelled cloethocarb or metaldehyde demonstrated that after rapid absorption in the foregut and crop the molluscicides were transported in the haemolymph to other organs such as the hepatopancreas, stomach, and skin. In the hepatopancreas, radioactivity was found mainly in the basophilic cells 1h to 10h after feeding and in the connective tissue (including macrophages) adjacent to the midgut gland tubules after l6h.

However, the sites and pathways of absorption and distribution of different compounds are dependant on their individual properties, such as lipophilicity. For example, l6h after feeding, the iron residue from chelate molluscicides was found in the resorptive cells of the hepatopancreas rather then in the basophilic cells, in a particular type of secondary lysosome.

The ability of slugs to store pollutants, particularly in the hepatopancreas, makes them useful indicators of environmental contamination, especially heavy metals. Experiments in which slugs were fed various metals at concentrations similar to those encountered in the field resulted in accumulations ofup to 71 µg Cd, 1169 µg Pb or 437 µg Zn per g dry wt of slug tissue.

Responses in different organs

Generally, in all the organs examined, certain cells were damaged or seriously discupted directly by the toxin immediately after its application. Such seriously affected cells were usually localized in "hot spots" in a particular part of the organ, and cells elsewhere in the organ usually maintained their function or showed only specific and limited responses to toxic impact.

Crop: With all the test materials lipid storage was reduced. The epithelial cells in control animals contained many lipid droplets, those from treated animals did not. In the molluscicide-treated animals, the activity of enzymes involved in transport processes (ATPases, alkaline phosphatases) was increased. When the toxic dose exceeded a certain limit a second stage of symptoms was observed where the epithelial cells were damaged and showed pathological symptoms.

Skin: Enhanced production of mucus and increased mucopolysaccharide synthesis was observed (this occurs in the stomach and the intestine to a lesser extent as well). Depending on the duration of exposure and concentration of toxin all types of mucocytes initially showed ultrastructural responses related to increased mucus production. Above a certain threshold, mucus cells (and other epithelial cells) showed signs of pathology. Metaldehyde caused an increase in ATPase activity especially at the bases of the mucus cells.

Hepatopancreas: In response to molluscicides, heavy metals, PCP and lindane, the resorptive cells ceased food absorption from the lumen and this was associated with an activation of intra- and/or extracellular digestion: Fusion of vacuoles and lysosomes increased and large vacuoles and secondary lysosomes dominated the cells. The lysosomes were not mainly near the cell apices as in controls, and after molluscicide exposure the pattern of acid phosphatases changed. Glycogen storage was generally reduced, microvilli shortened, and the number of pinocytotic vesicles or channels drastically reduced. The activity of non-specific esterases especially in the lumen, and of alkaline phosphatases at the base of the tubules was increased.

In the basophilic cells organic and inorganic pollutants (which were both located within these cells by tracing techniques) caused structural modification of organelles (especially endoplasmic reticulum), which was correlated with an induction of enzymes involved in biotransformation (NTR, AHH) and in cellular transport (ATP, AP). Storage products in the basophilic cells were significantly reduced.

Stress protein exxression

The stress protein (hsp70) level increased by up to ten times compared to control animals in slugs treated with all the compounds except γ-HCH. A dose-response relationship was observed with all the heavy metal and PCP treatments, with increased proteotoxicity induced even by low concentrations of toxins. When very high levels were applied however, the relationship broke down and the hsp70 level declined in acutely poisoned slugs, as reported in other soil invertebrates (Guven *et al.*, 1994, Eckwert *et al.*, in press). This effect might explain the low hsp70 levels in slugs treated with γ-HCH.

DISCUSSION

The results obtained after exposure to both organic and inorganic chemicals suggest that there are three main groups of cellular response: **1) Responses indicating cell damage** such as cellular pathology, a decrease in enzyme activies or a reduction in the level of stress protein (both the latter probably caused by a breakdown in the protein synthesis apparatus). These reactions are widespread in target cells (and in non-target cells as well with acute poisoning) and are the desired effects of molluscicides. Slugs rnight survive chronic exposure as long as cell damage remains below a critical level and is confined to restricted portions of their organs which can then still function adequately. Such conditions are frequently encountered in sublethally polluted environments. **2) Responses associated with defence or repair mechanisms**. The most obvious of these is enhanced mucus secretion by the skin, although it occurs in the stomach, intestine and parts of the genital tract (Triebskorn & Ebert, 1989). The copious mucus could serve to dilute the toxin and sometimes, for example in the digestive tract where the pH is 4 -5, even detoxify it if it is pH sensitive (Triebskorn, unpublished). Exposure to organic toxins often induces biotransformation enzymes, and exposure to metals can lead to the formation of spherites (e.g. Hopkin, 1989) or increased expression of metal binding proteins or metallothioneins (Dallinger *et al.* , 1989; Berger *et al.* ,

1995). Another major response in this group is the expression of stress proteins which indicate proteotoxicity and can refold malfolded proteins to some extent in affected cells (e.g. Hightower, 1991). All these responses begin or are enhanced in order to get rid ofthe toxin or to limit damage, and in the event of long-term chronic exposure slugs can keep them activated over long periods; they are therefore useful as “biomarkers" for chernical pollution. To molluscicides, these reactions are obstacles to be overcome to improve efficacy. The final group of reactions are **3) responses associated with energy production**. All the processes in group 2 are energetically expensive. For example, even under unpolluted conditions, a limpet expends about a quarter of its daily energy budget in production of pedal mucus for adhesion and locomotion (Davies *et al.*, 1990). A large amount of extra energy is needed to fuel the repair and defence mechanisms described above, and it may be provided by intensified intracellular digestion in the hepatopancreas which leads to the increased fusion of vacuoles, increased activity of enzymes involved in intra- and extracellular digestion, depletion of storage products in the crop and hepatopancreas, and increased activities of enzymes involved in transport.

It may be that the responses in the three groups described above are interdependent, and the limiting factor for tolerance of chemical stress is the energy available to maintain the defence mechanisms. Fig. 1 summarizes this hypothesis and illustrates how the responses in group 1 (represented by "cellular pathology") and group 2 (represented by "stress proteins") may be affected differently by the finite "energy" available in both chronic and acute toxic conditions. In the environment, chronic exposure conditions could have a significant effect on an entire ecosystern, since in order to compensate for a condition of permanent chemical stress many organisms may have to keep repair and defence mechanisms continually activated, and invest large amounts of energy into limiting cell damage. This energy expenditure will build up a considerable deficit and so the animals' requirement for energy from feeding is consequently increased and any short-term food shortages which would be otherwise tolerable may not be survived. Energy for reproductive processes may also be limited and chronic exposure to pollutants often results in reduced reproductive success (summarized by Kammenga, 1996). The balance of the community of decomposers in polluted soils may ultimately be significantly altered, and chemical pollution or extended intensive chemical crop protection treatments provide a strong selective pressure for resistant genotypes which, in the long term, bodes ill for chemical pest control.

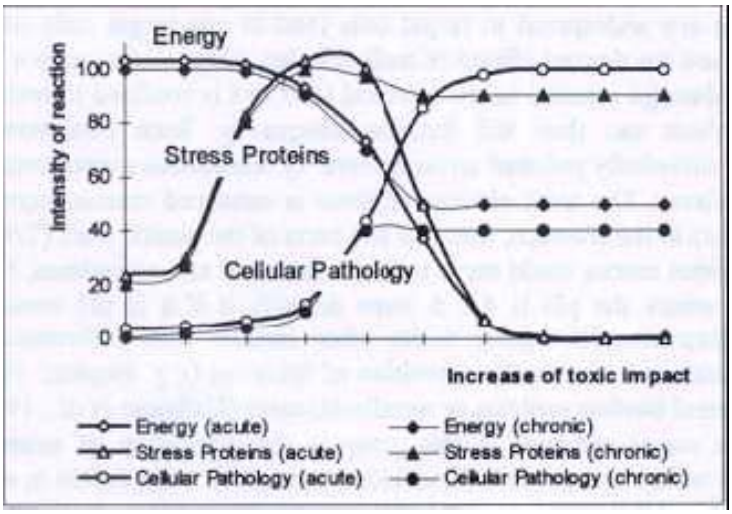

Fig.1: Hypothetical response to acute and chronic toxic exposure of stress proteins (hsp70) and cellular pathology in relation to energy budget.
"Increased toxic impact" means longer exposure to moderate toxin concentration in chronic exposure; but under acute conditions high concentrations can only be tolerated for a short time. The timescales for the two conditions are therefore different which is not reflected in the plot.

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