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THE IMPACT OF MOLLUSCICIDES ON ENZYME ACTIVITIES IN THE HEPATOPANCREAS OF *DEROCERAS RETICULATUM* (MÜLLER)

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ABSTRACT

The influence of three commercial molluscicide pellets, Cloethocarb, Mesurol, and Spiess Urania 2000, on the activities of six enzymes in the hepatopancreas of *Deroceras reticulatum* were investigated by light and electron microscope histochemisty as well as by photometric studies. In the digestive cells, enzymes catalyzing energy-producing digestive processes (non-specific esterases and acid phosphatases) are induced, while, in the crypt cells, enzymes related to energy-consuming metabolic pathways often involved in detoxification (alkaline phosphatase, and NADPH-neotetrazoliumreductase) are activated.

Key words: *Deroceras,* molluscicides, enzymes, hepatopancreas.

INTRODUCTION

Xenobiotics are known to interact directly with enzymes (Wilkinson, 1976; Dauterman & Hodgson, 1990; Eldefrawi & Eldefrawi, 1990). Carbamates, for example, bind to esterases (Gordon & Eldefrawi, 1960; Tegelsström & Wahren, 1972) or, more specifically to cholinesterases as competitive inhibitors (Metcalf & Fukuto, 1965; Young & Wilkins, 1989; Eldefrawi & Eldefrawi, 1990). Furthermore, toxic substances can influence the homeostasis of the cell by destruction of such cellular components as mitochondria (Triebskorn, 1988). In general, the destruction of cellular structures may result from an interaction between lipophilic chemicals and membranes (Sparks, 1972; Trump et al., 1981; Cascorbi & Pauli, 1990). The cell is able to react to alterations in cell homeostasis either by intensification of special metabolic pathways or by induction of particular enzymes. For example, enzymes normally catalyzing oxidative digestive processes or being involved in steroid metabolism might be used in detoxification through oxidation (den Besten et al., 1990). The activation of such oxidative processes by xenobiotics has often been described for vertebrates (Stohs et al., 1976; Hinton et al., 1978; Kagan, 1988), but is also mentioned for invertebrates (Lee, 1981; Widdows et al., 1981; Stegeman, 1985; Livingstone, 1988).

In the present study, enzyme activities in the hepatopancreas of *Deroceras reticulatum* were studied. In this organ, one cell type in particular, the crypt cell, which is also called basophil cell, seems to possess a special function in detoxification of pollutants (Simkiss & Mason, 1984). This assumption is supported by my own investigations in which it has been shown that radioactive material is present in the crypt cells shortly after feeding the animals with ^{14}C-labeled Cloethocarb (Triebskorn et al., 1990). Furthermore, Cajaraville et al. (1990) observed an increase in the number of basophil cells after exposing animals to petrol hydrocarbons.

In the present study, first, molluscicide-induced alterations in the activity of lysosomal enzymes involved in intracellular digestion (esterases and acid phosphatases) have been investigated. Alterations in lysosomal hydrolases induced by different kinds of stress have been described by Moore (1976), Moore & Halton (1977), Moore (1982), Moore et al. (1982), Cajaraville et al. (1989).

Second, enzymes related to transport processes (alkaline phosphatases and ATPases) were studied. Banna (1980) demonstrated that after application of the molluscicide Frescon the activity of the alkaline phosphatase in the hepatopancreas of *Bulinus truncatus* is higher than in control animals.

Finally, two enzymes known to be involved in oxidative detoxification (NADPH-neotetrazolium reductase and arylhydrocarbon hydroxylase) were analyzed.

TABLE 1. Time spans between the first ingestion of the different molluscicides and dissection. L: Light microscopy; E: Electron microscopy; P: Photometric measurment

	Cloethocarb				Mesurol	Metaldehyde
	2%	0.1%	0.01%	0.001%	4%	4%
Non-spec. esterase						
Acid phosphatase				—		
				—		
				—		
Alk. phosphatase		5hL	5hL	—	5hL	5hL
		—	—	—	—	—
		—	—	—	14hE	—
ATPase		—	—	—	1.5hL	4hL
		—	—	—	5hL	5hL
NADPH-neotetra-zoliumreductase		5hL	5hL	3wL		5hL
Arylhydrocarbon-hydroxylase	5hP				5hP	5hP

MATERIALS AND METHODS

Laboratory-reared *Deroceras reticulatum* were fed molluscicides, which were applied either as commercial pellets or as a self-made wheat-bran agar formulation containing different concentrations of the molluscicide agents. Control animals were fed wheat-bran agar. The following substances were used:

Mesurol: commercial pellets containing 4% of the effective substance 4-(methyl-thio)-3,5-xylyl-methyl-carbamate.

Spiess-Urania 2000: commercial pellet containing 4% metaldehyde.

Cloethocarb: wheat-bran agar containing 2%, 0.1%, 0.01% or 0.001% of 2-(2-chloro-1-methoxy-ethoxy-)phenyl-N-methylcarbamate.

The animals were dissected at different times after the first ingestion of the molluscicides. The duration of exposure is illustrated in Table 1.

For light microscope, histochemical enzyme tests, the digestive system was isolated and the hepatopancreas was either frozen in isopentan, which was cooled in liquid nitrogen, or was fixed for 1 h in 2% glutaraldehyde (dissolved in 0.01 M cacodylic buffer, pH 7.4). Fixed material was embedded without being dehydrated in HistoResin.

For enzyme tests on the electron microscope level, vibratome-cut sections of the hepatopancreas (of about 100 μm) were fixed in a 2% glutaraldehyde solution in cacodylic buffer (0.01 M, pH 7.4) for 45 min (at 4°C). Afterwards, they were rinsed in the same buffer (in the test for esterases 10% dimethylsulfoxide was added), and then they were transfered into the incubation medium. After incubation, the tissues were postfixed in reduced osmium (Karnovsky, 1971), stained en bloc with uranylacetate, dehydrated, and embedded in Spurr's medium (Spurr, 1969).

Ultrathin sections of 100-200 nm were cut on a Reichert-OM-U2 ultramicrotome and examined in a Zeiss EM 9 without further staining.

For photometric measurements of enzyme activities, the animals were dissected, the digestive tract and the hepatopancreas were isolated, the gut content was removed, and finally the tissues were frozen at −80°C in a deep-freezer. For arylhydrocarbonhydroxylase, fresh tissues (the hepatopancreas not removed from the digestive tract) were used. The tissues were put in a buffer specific for the enzyme tested, homogenized either in a Polytron-Kinematica potter or in a hand-potter (for arylhydrocarbonhydroxylase), and centrifuged in a Sorvall RC 2-B centrifuge. For the different enzyme tests, saturation curves were obtained in a LKB Biochrom Ultraspec Plus or in a Beckman DU-6 spectrophotometer.

Finally, the enzyme activity could be calculated using the following formula:

$$c = \frac{\Delta E/min \times v}{\varepsilon x d x v} \quad [mUnits/ml]$$

c: enzyme activity
ΔE: difference of extinction
V: test volume (content of the cuvette, 1ml)
v: sample volume (0.02 ml)
d: diameter of the cuvette (1 cm)
ε: coeffizient of extinction ($cm^2/\mu mol$)

The following methods were used

Non-specific esterases (NE)

Light microscope-HistoResin sections
Method: Lojda et al. (1976)
Substrate: natriumdisphosphate
Electron microscope
Method: Bell & Barnett (1965)
Substrate: thioacetoacid

Acid phosphatases (AcP)

Light microscope-HistoResin sections
Method: Werner (1986)
Substrate: naphthol-AS-biphosphate
Electron microscope
Method: Robinson & Karnovsky (1983)
Substrate: Na-β-glycerophosphate
Photometric measurement
Method: Bergmeyer (1970)
Substrate: p-nitrophenylphosphate

Alkaline Phosphatases (AlP)

Light microscope-HistoResin sections
Method: Werner (1986)
Substrate: naphthol-AS-MX-phosphate
Electron microscope
Method: Robinson & Karnovdky (1983)
Substrate: Na-β-Glycerophosphate
Photometric measurement
Method: Bergmeyer (1970)
Substrate: p-nitrophenylphosphate

Na^+-Ka^+-ATPase (ATP)

Light microscpoe-cryosections
Method: Wachstein & Meisel (1957)
Substrate: ATP -Na^+-salt

NADPH-neotetrazoliumreductase (NTR)

Light microscope-cryosections
Method: Lojda et al. (1976), Bayne et al. (1985)
Substrate: NADPH

Arylhydrocarbonhydroxylase (AHH)

Photometric measurement
Method: Collodi et al. (1984)
Substrate: diphenyloxazole

RESULTS

In the following, molluscicide-induced alterations of enzyme activity in the hepatopancreas of *Derocras reticulatum* will be described. The results from the enzyme histochemical studies are confined to reactions in the three cell types of the hepatopancreas: the digestive cells, the crypt cells, and the excretory cells. For the photometric measurements, the enzyme activity in the hepatopancreas will be compared with that in the alimentary tract, except for the arylhydrocarbonhydroxylase, because for this test, the hepatopancreas had not been removed from the digestive tract.

Non-specific Esterases

Light microscopy: Control. Most of the reaction product is localized in small vesicles in the apical half of the digestive cells (Fig. 1). Less intense reaction can be observed in the cytoplasm.

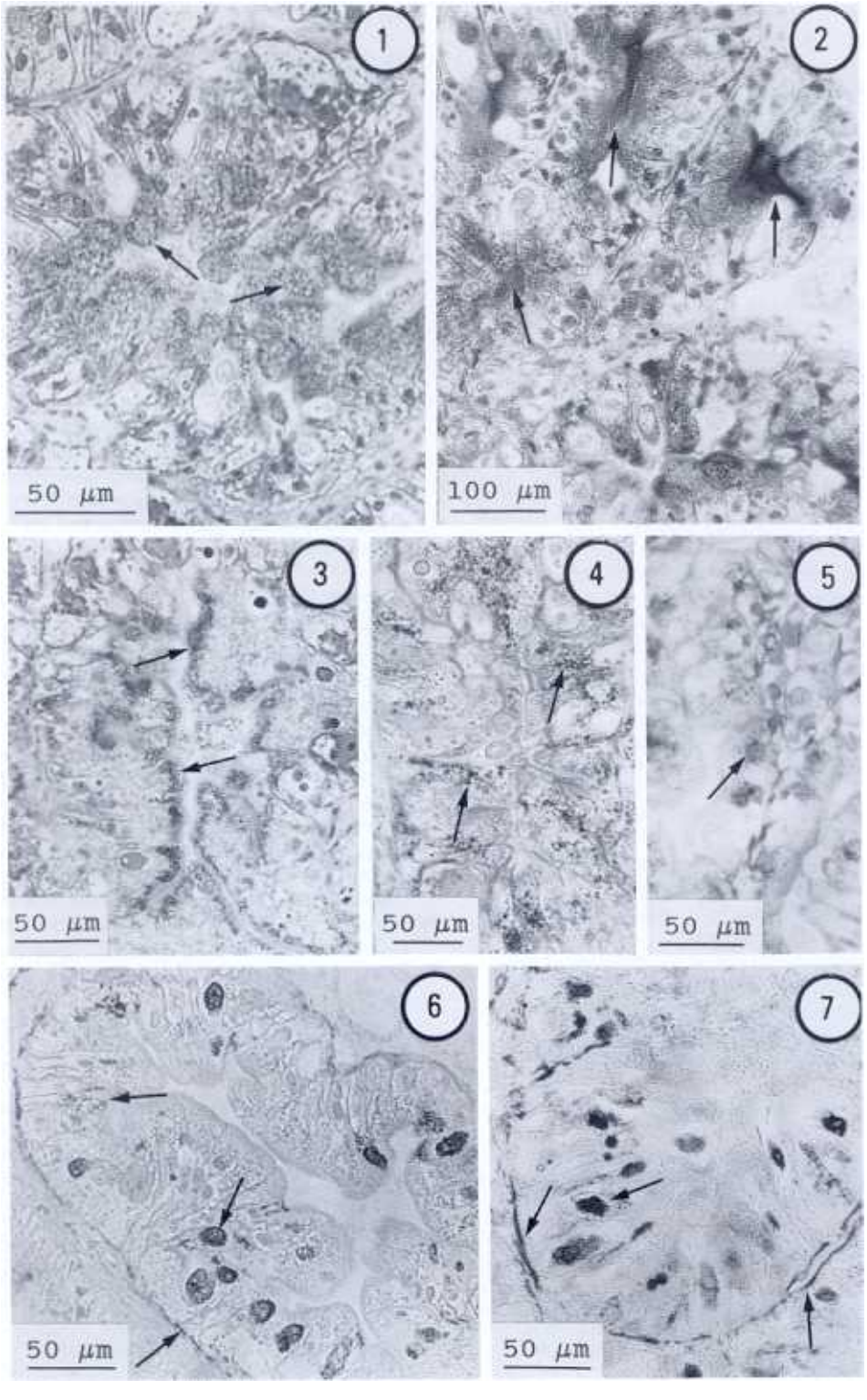
Cloethocarb. With all Cloethocarb concentrations, but especially with the 0.01% oral dose (OD), the reaction of esterases in the cytoplasm of digestive cells is stronger than in the controls. Large amounts of reaction product can be found in the tubular lumen (Fig. 2).

Mesurol. After ingestion of Mesurol, the reaction in the digestive cells is more intense than in the controls. The reaction products are dispersed throughout the cytoplasm of the cells.

Metaldehyde. The cytoplasm of the digestive cells stained more intensely than in the controls.

Electron microscopy: Control. Reaction product is localized in the cytoplasm and on the microvilli of the digestive cells (Fig. 13), and in the endocytotic channels and vesicles originating between them (Fig. 14). Vesicles in the apical half of the cell show enzyme activity, as well. Small amounts of precipitate can be found dispersed in the cytoplasm of digestive and crypt cells. Furthermore, small vesicles in the crypt cell stained intensely.

Cloethocarb. After application of 2% or 0.1% OD, the reaction of non-specific ester-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ases in the cytoplasm as well as on the microvilli and cell bases of digestive and crypt cells is stronger than in controls (the last two reactions can also be observed with 0.01% OD) (compare Figs. 13, 14 with 15, 16). Except for 0.01% OD, precipitate in the endocytotic channels and vesicles of the digestive cells is not observable.

Acid phosphatase

Light microscopy: Control. Reaction product is localized distinctly in small vesicles in the most apical parts of the digestive cells (Fig. 3). The enzyme activity in the cytoplasm of the digestive, crypt, and excretory cells is less intense.

Cloethocarb. After poisoning the animals with Cloethocarb in concentrations higher than 0.01%, only few reaction of the enzyme can be observed in the digestive cells (Fig. 5). After 0.01% OD, only few vesicles in the middle of the digestive cells are stained.

Mesurol. The reaction after uptake of Mesurol is less intense than in controls, but more intense than after Cloethocarb ingestion. In some cells, precipitate can be observed in vesicles in the middle of the digestive cells as well as dispersed over the cytoplasm (Fig. 4).

Metaldehyde. Phosphatase activity in the digestive cells was reduced after poisoning the animals with metaldehyde. However, the reaction is more intense than after ingestion of the two carbamates. In contrast to the controls, reaction product can be found in the basal parts of the digestive cells.

Electron microscopy: Control. In the digestive cells, reaction product is localized in small vesicles that are often attached to large vacuoles or lipid droplets in the apical half of the cells (Fig. 17). Little enzyme activity can be found on the microvilli of digestive, crypt, and excretory cells.

Cloethocarb: As soon as 1 h after poisoning the animals with 2% Cloethocarb, but especially after 14 h, the most striking enzyme activity can be observed in the endoplasmic reticulum, the Golgi apparatus (Figs. 18, 19, 20), and in the basal labyrinth of the crypt cells. Large amounts of precipitate can also be found in the cytoplasm and in small vesicles in close contact to dictyosomes and endoplasmic reticulum. The enzyme reaction in this cell type is more intense than in the controls. In the digestive cells, enzymatically active vesicles can be found in the basal half of the cells. The microvilli of digestive and excretory cells show intense enzyme activity. After 1 h, enzyme activity is also present in the lumen of the gland tubules, especially in regions where cells are extruded from the epithelia. Furthermore, especially after 14 h, the hemolyph space is distinctly stained.

Mesurol: 14 h after the first ingestion of Mesurol, large numbers of heavily destroyed digestive and crypt cells can be found, with precipitate dispersed throughout the cytoplasm. Phosphatase activity in the endoplasmic reticulum, the Golgi apparatus, and the vesicles in the crypt cells is as intense as after Cloethocarb poisoning. In the digestive cells that are still intact, reaction product is localized on the microvilli and in small vesicles between the microvilli. The hemolymph space is heavily stained.

Photometric measurement: Figure 25 clearly demonstrates that the activity of acid phosphatases in the hepatopancreas (9.43) is nearly twice as high as in the alimentary tract (4.71). Whereas the enzyme activity in the alimentary tract was not influenced by the molluscicides, all of the molluscides tested induced a similar reduction in acid phosphatase activity in the hepatopancreas by approximately 35%.

Alkaline Phosphatase

Light microscopy: Control. In the most basal parts of the epithelial cells, a small layer showing enzyme activity can be observed.

FIGS. 1–7. Light microscope staining for non-specific esterases (NE), acid (AcP) and alkaline (AIP) Phosphatases).
1. NE, control. Enzyme reaction in vesicles in the apical half of the digestive cells (arrows), ×380.
2. NE, 2% Cloethocarb, 5 h. Increased enzyme activity in the lumen of the tubules (arrows), ×130.
3. AcP, control. Positive reaction in vesicles in the most apical parts of the digestive cells (arrows), ×240.
4. AcP, Mesurol, 5 h. Minimal precipitate in the middle of the digestive cells (arrows), ×200.
5. AcP, 2% Cloethocarb, 5 h. weak enzyme reaction in the digestive cells (arrows), ×250.
6. AIP, 0.01% Cloethocarb, 5 h. Reaction product on the cell bases, in apical lying vesicles of the digestive cells and in excretory granules (arrows), ×250.
7. AIP, 2% Cloethocarb, 5 h. Strong reaction on the cell bases and in excretory granules (arrows), ×250.

Furthermore, reaction product is localized in small vesicles in the apical half of the digestive cells and in the excretory cells.

Cloethocarb. After uptake of 2% or 0.1% Cloethocarb, the reaction in the most basal parts of the cells is more intense than in controls or after 0.01% Cloethocarb, which is similar to that in controls (compare Figs. 6 and 7). Vesicles in the digestive cells stain less and reaction in the excretory cell is more distinct than in controls.

Mesurol: The reaction can be compared with that occuring after Cloethocarb intoxication. The most striking reaction is to be found in the excretory cells.

Metaldehyde. After ingestion of the metaldehyde-containing pellets, reaction in the basal parts of the cells, in the excretory cells and in the lumen of the tubules is more intense than in the controls.

Electron microscopy: Control. While on the microvilli of digestive and crypt cells only few reaction products could be found, the apical surfaces of the excretory cells reveal intense phosphatase activity. In the digestive and crypt cells, the cisternae of the endoplasmic reticulum located close to the cell surface are distinctly stained. Furthermore, small phosphatase-positive vesicles are present in the basal parts of the crypt cells. Precipitate can also be found in the excretory vacuoles. Relatively little reaction product can be observed dispersed throughout the hemolymph.

Cloethocarb. After 1 h, but more intensely after 14 h, the most striking reaction of alkaline phosphatase can be found in the endoplasmic reticulum of both the digestive and the crypt cells (Figs. 21, 22). Precipitate is also present in cis-face cisternae of the Golgi apparatus and in basally lying vesicles of the crypt cells (Figs. 23, 24). In the digestive cells, reaction product often surrounds lipid droplets. Large amounts of precipitate can be found in the hemolymph space and in the tubular lumen.

Mesurol. Similar to ingestion of Cloethocarb, the enzyme activity in the endoplasmic reticulum of digestive and crypt cells is intensified. The most distinct reaction can be observed in cisternae lying next to the cell surface. Furthermore, large amounts of precipitate are present in small vesicles in the basal parts of the crypt cells and in the hemolymph space.

Photometric measurement: Figure 26 shows that in control animals the activity of alkaline phosphatase in the hepatopancreas (2.35) is lower than in the alimentary tract (3.66). In both parts of the digestive system, the phosphatase activity is activated by molluscicides. After poisoning the animals with metaldehyde, the enzyme activity in the hepatopancreas is more than twice as high as in the controls (6.53). After application of the two carbamates, it is increased to 3.97 (Cloethocarb) or 4.33 (Mesurol).

ATPase

Light microscopy: Control. Enzyme activity can especially be found in the basal parts of the epithelial cells, as well as in the muscle and nerve tissue underlying the epithelia. Positively reacting vesicles are also present in the apical parts of the digestive cells and in vesicles in the crypt cells (Fig. 8).

Cloethocarb. The reaction in the basal parts of the epithelial cells and in the muscle and nerve tissue is slightly reduced. Large amounts of reaction product can be found in the apical parts of digestive cells, either localized in vesicles or dispersed throughout the cytoplasm.

Mesurol. The reactions are similar to those after Cloethocarb ingestion (Fig. 9).

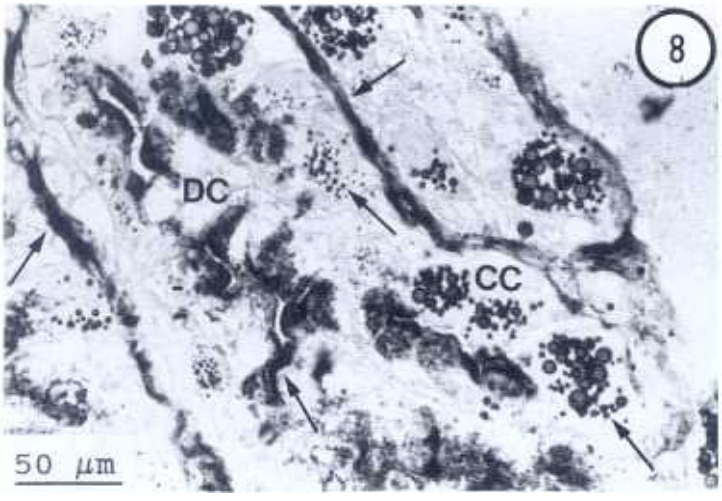
Metaldehyde. Enzyme activity in the apical parts of the digestive cells is more intense than in controls. Reaction product is distinctly localized in vesicles and on the microvilli border (Fig. 10).

NADPH-Neotetrazoliumreductase

Light microscopy: Control. In the control animals, enzyme activity can be found in the crypt cells, especially on the cell apices (Fig. 11). The apices of the digestive cells are free of precipitate.

Cloethocarb. After uptake of 2% or 0.1%

FIGS. 8–10. Light microscope staining for ATPase (ATP).
8. ATP, control. Strong reaction on the cell bases, in vesicles of crypt (CC) and digestive cells (DC) as well as in muscle tissue, ×260.
9. ATP, Mesurol, 5 h. Strong reaction on the cell apices and in muscle tissue, ×400.
10. ATP, Metaldehyde, 4 h. Strong enzyme activity on the microvillous border and in apical lying vesicles of the digestive cells as well as in muscle tissue, ×260.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DC
CC
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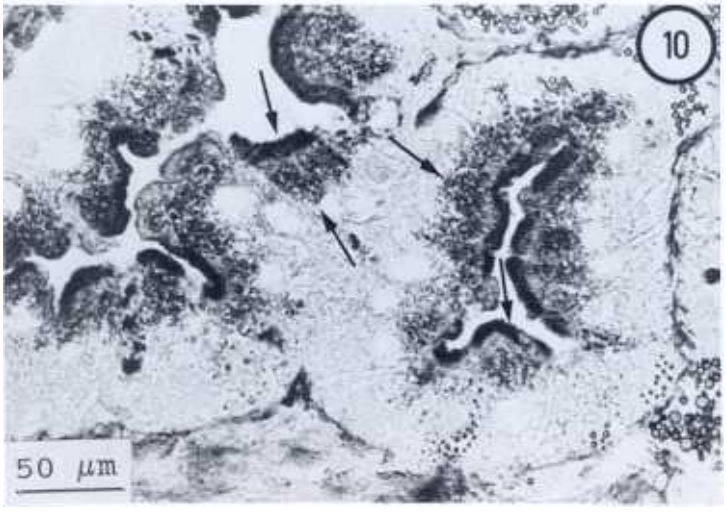
9
20 µm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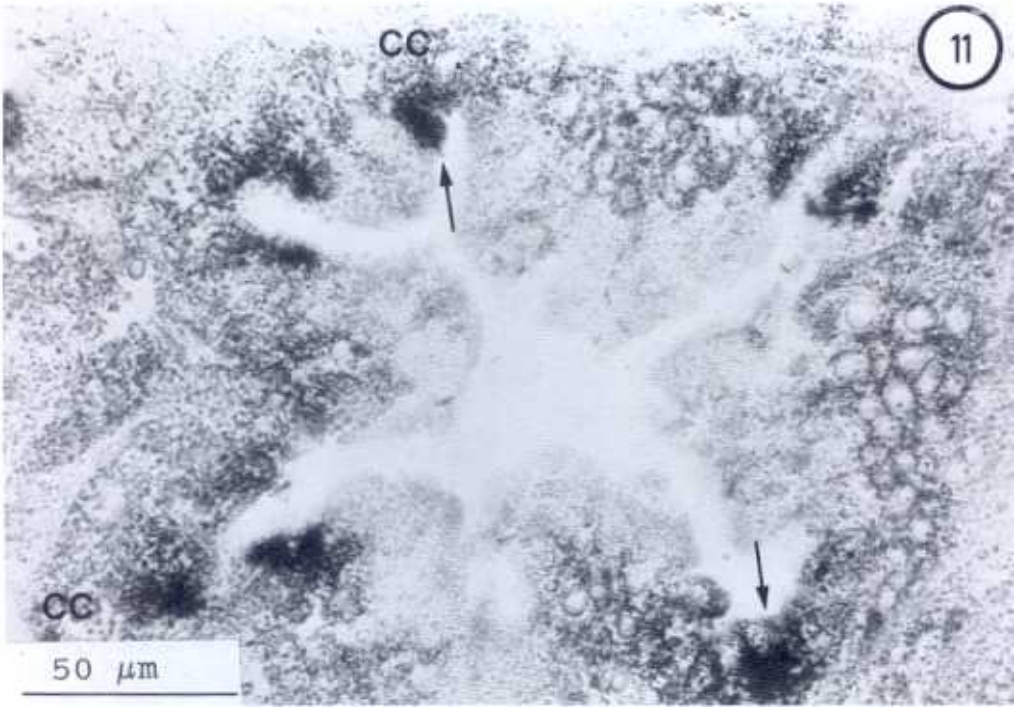

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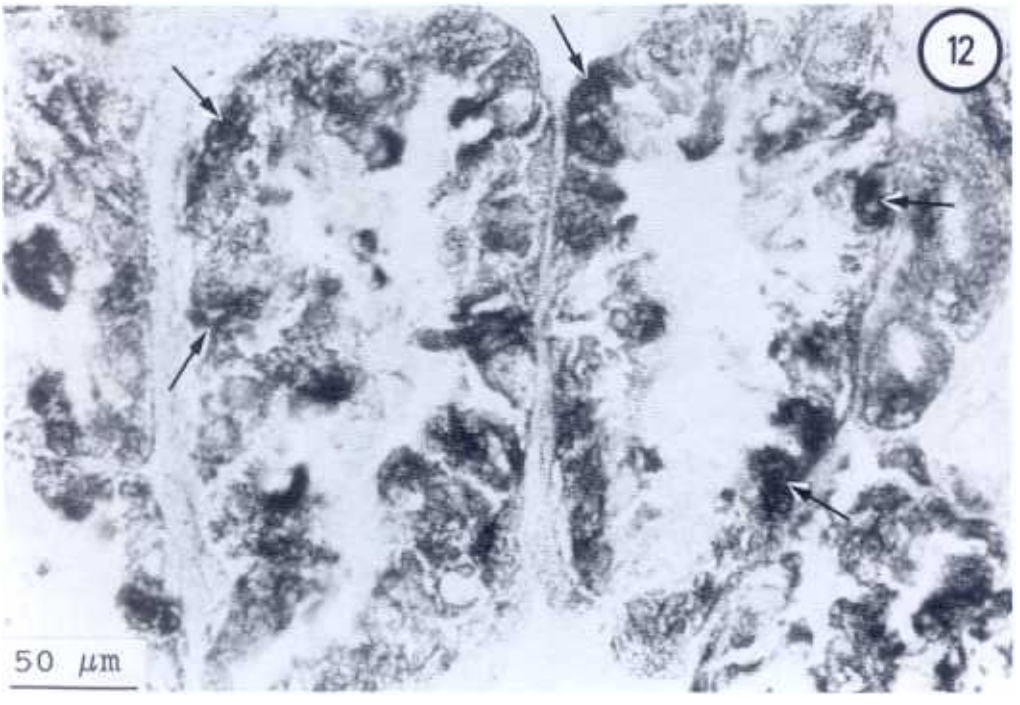

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Cloethocarb, the enzyme activity in the hepatopancreas is stronger than in the controls. Especially in the crypt cells, reaction product is dispersed throughout the cytoplasm (Fig. 12). After ingestion of sublethal concentrations (0.001%), the reaction in the crypt cells is more intense than after 5% or 0.1%.

Metaldehyde: The reaction can be compared with that after ingestion of sublethal Cloethocarb concentrations.

Arylhydrocarbonhydroxylase

Photometric measurement: Figure 27 demonstrates that the enzyme is clearly activated by metaldehyde (from 5.19 to 10.39). Mesurol induces a slight increase (to 7.7), whereas the enzyme activity is not influenced by Cloethocarb (5.3).

DISCUSSION

In the present study, it is shown that poisoning slugs with molluscicides induces modifications in the activity of several enzymes. Alterations in enzyme activity can be correlated with molluscicide-induced changes in ultrastructure of the cells in the hepatopancreas of *Deroceras reticulatum* described previously (Triebskorn, 1989; Triebskorn & Künast, 1990).

In a first step, alterations of hydrolases were investigated. The impact of molluscicides on these intracellular digestive enzymes is very important, as they are involved in essential metabolic pathways during intracellular digestion, breaking down ester compounds by hydrolysis. Because it has already been reported by Tegelström & Wahren (1972) that carbamates not only inhibit cholinesterases but influence esterases in general, the affect of Cloethocarb on non-specific esterase activity was examined. Non-specific esterases is the term used for a group of enzymes that includes carboxylesterases, arylesterases and acetylesterases. An inhibition of these non-specific esterases could be observed only in the cells of the alimentary tract (e.g. in the oesophagus, crop stomach and gut) (Triebskorn, unpublished). In the hepatopancreas, the enzyme activity in the cytoplasm of the cells was stronger after application of the molluscicide than in controls. This might be related to the fact that several types of esterases exist in the different parts of the digestive system with different sensitivities to carbamates.

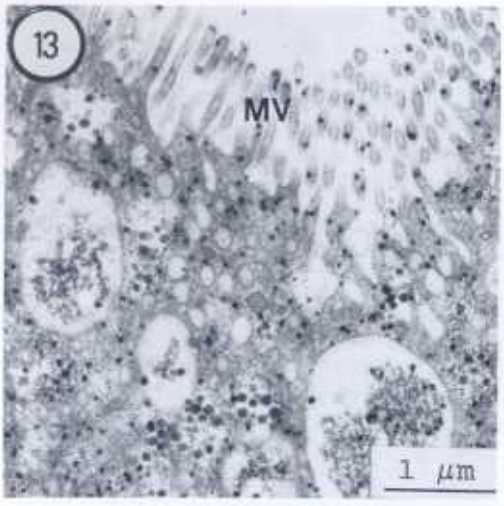
After carbamate poisoning, the high activity of esterases in the cytoplasm of digestive cells and the lack of reaction product in the vesicles might be due to the destruction of vesicle membranes, as described by Triebskorn & Künast (1990). Esterases normally found in membrane-bound compartments are set free into the cytoplasm and catalyze reactions leading to autolysis. Such autolytic processes are often discussed for terrestrial and marine invertebrates as a result of stress-induced instability of lysosome membranes (Moore, 1976; Oxford & Fish, 1979; Cajaraville et al., 1989). However, after metaldehyde ingestion, large amounts of non-specific esterases can be found in the digestive cells, even though the lysosomal system is not as damaged as after carbamate poisoning (Triebskorn, 1989).

In general, the results of the electron microscope test in control animals make evident that some of the non-specific esterases localized in vesicles in the apical parts of the digestive cells of the hepatopancreas are not produced by these cells, but are resorbed by pinocytotic processes. This can be assumed from the fact that no Golgi apparatus could be found producing these esterase-positive vesicles and that high enzyme activity is present in the lumen of the tubules as well as in pinocytotic vesicles and channels.

The presence of non-specific esterases in vesicles of the crypt cells might also be due to intracellular digestive processes. Especially because crypt cells are thought to be involved in detoxification of such foreign compounds as heavy metals (Simkiss & Mason, 1984), a functional role of esterases in detoxification might be possible. The role of esterases in detoxification of carbamates is mentioned by Gordon & Eldefrawi (1960), who speak of special "carbamate-esterases."

As a second intracellular digestive enzyme-system, the acid phosphatases, known as key enzymes in primary lysosomes (Goldfischer et al., 1964), were examined. Enzymes of this group are able to catalyze the breakdown of

FIGS. 11, 12. Light microscope staining for NADPH-neotetrazolium reductase (NTR).
11. NTR, control. Reaction on the apices of crypt cells, ×480.
12. NTR, 0.1% Cloethocarb 5 h. Increased enzyme activity in the crypt cells, ×300.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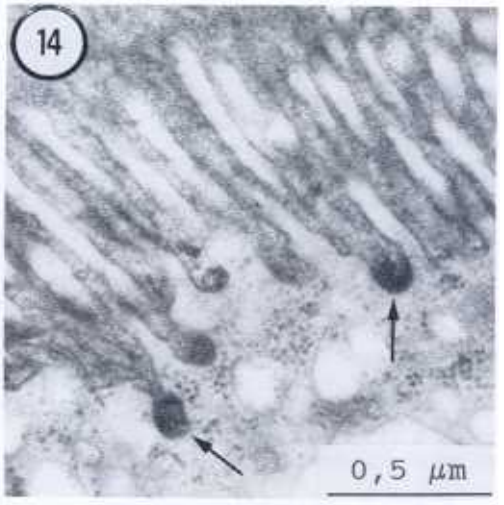
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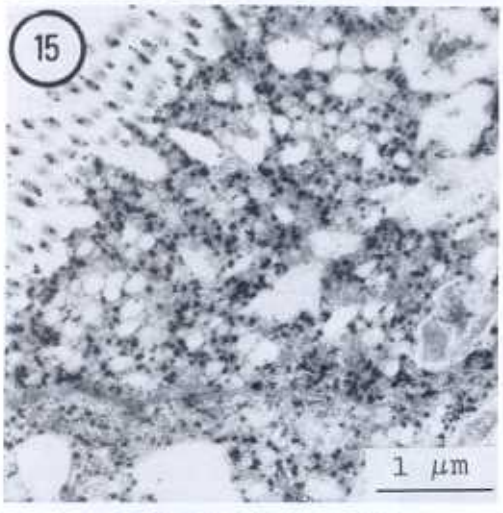
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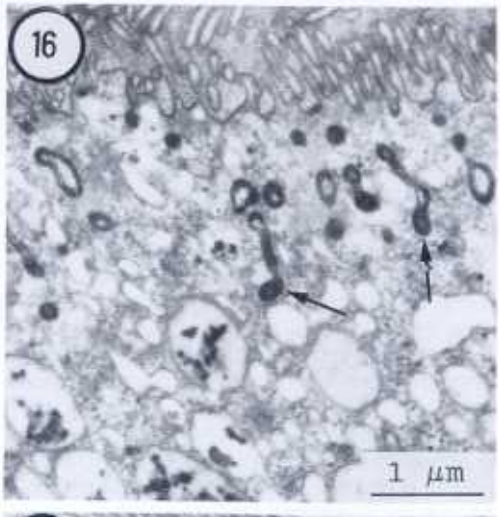
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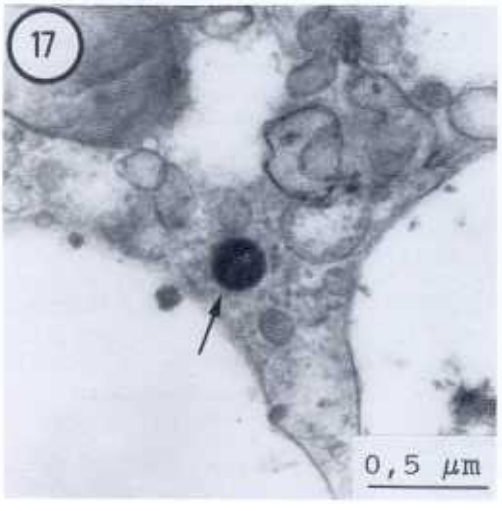
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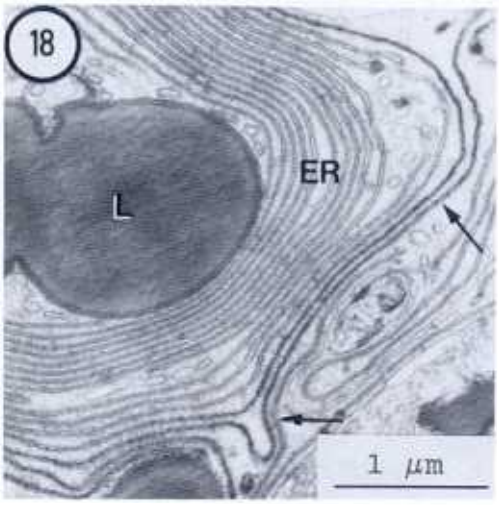
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esterbonds in orthophosphate esters under acid conditions and are involved in the attack of pyrophospate bonds. They act additionally as transphosphorylases (Lojda et al., 1976).

The distribution of this enzyme activity in the three cell types of the hepatopancreas agrees well with that described by Sumner (1969) for the midgut gland cells of *Mytilus edulis* and *Helix pomatia* and by Bowen (1970) for those of *Arion ater.*

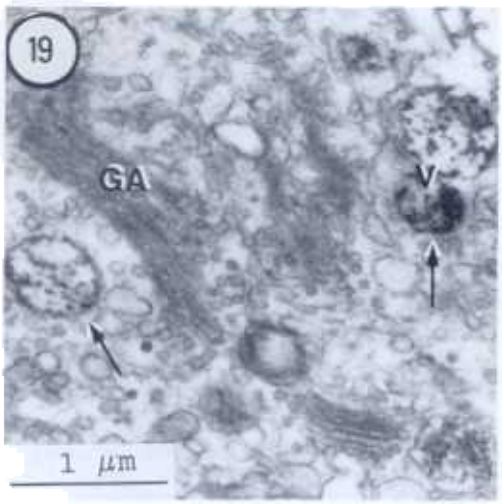
In the crypt cells, Golgi cisterns, which stained strongly for the enzymes, are similar to those described as rigid lamellae, and large phosphatase-active vesicles seem to be condensing vacuoles (Novikoff et al., 1977; Hand & Olivier, 1977).

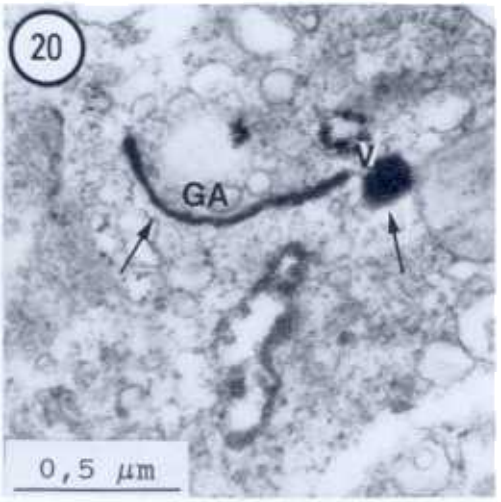
In the vacuolar system of the digestive cells, the positive-reacting vesicles might be primary lysosomes. It had been expected that molluscicide ingestion, leading to lysosomal instability, would result—as in the case of esterases—in increased phosphatase activity in the cytoplasm and reduced activity in the vesicles. This turned out not to be true. The light microscope tests clearly demonstrate that after Cloethocarb poisoning, phosphatase activity is totally diminished, where as after Mesurol and metaldehyde a little activity can still be observed in basally located vesicles. This speaks in favour of two phenomena: first, the membranes of primary lysosomes seem to be less unstable than those of secondary lysosomes, which contain non-specific esterases and break down under stress conditions; second, acid phosphatases seem to catalyze reactions at the beginning of intracellular digestion shortly after the fusion of primary lysosomes with digestive vacuoles or secondary lysosomes. Molluscicides induce the fusion activity (eventually due to an impact on the cytoskeleton) and therefore accelerate phosphatase-catalyzed primary digestive processes. This hypothesis is also supported by the present photometric measurements, which demonstrate the importance of the hepatopancreas in digestive processes catalyzed by acid phosphatase. The results of electron microscope studies, however, do not correspond well with the molluscicide-induced reactions observed in light microscope studies. After taking up Cloethocarb, the digestive cells show small vesicles with distinct phosphatase activity in the basal parts of the cell. Furthermore, the strong reaction on the microvilli and in the tubular lumen could not be found in light microscope studies. The differing results, obtained by different methods, might be due to the fact that in all these tests different substrates for acid phosphatases were used, and more or less different types of acid phosphatases may have been stained. The substrate specifity of enzyme groups and the problems that may arise if results obtained by different methods are compared are also emphasized by Newell (1977), Oxford & Fish (1979), and Bowen (1981).

As enzymes possibly involved in transport processes (Lojda et al., 1976), alkaline phosphatases and ATPases were investigated. Alkaline phosphatases break down ester compounds of orthophosphate acids under alkaline conditions between pH 9.2 and 9.8 (Lojda et al., 1976). A special type of alkaline phosphatase with a pH optimum of 7.5 is capable of catalyzing the hydrolytic break down of ATP. This is the cell membrane ATPase, the activity of which depends on the presence of Na^+- and K^+- ions.

All molluscicides tested induced an increase in the activity of the alkaline phosphatases in the cells of the hepatopancreas. This was evident in the light and electron microscope tests, as well as in the photometric measurements. The increased activity of the enzyme, especially on the basal cell surfaces, might be correlated with an intensified transport of the molluscicides from the hemolymph space into the cells of the hepatopancreas. The presence of ATPase in these regions of

FIGS. 13–18. Electron microscope investigations for non-specific esterases (NE) and acid phosphatases (AcP).
13. NE, control. Enzyme reaction in the cytoplasm and on the microvilli (MV) of a digestive cell, ×14200.
14. NE, control. Reaction product in pinocytotic channels and vesicles of a digestive cell, ×42000.
15. NE, 2% Cloethocarb, 6 h. Strong enzyme activity in the cytoplasm of a digestive cell, ×14200.
16. NE, 0.01% Cloethocarb, 6 h. Enzyme activity in pinocytotic channels of a digestive cell, ×14200.
17. AcP, control. Vesicles with enzyme activity in a digestive cell, ×31300.
18. AcP, 2% Cloethocarb, 14 h. Endoplasmic reticulum (ER) with enzyme activity surrounding lipid droplet (L). ×19300.


19
GA
V
1 µm


20
GA
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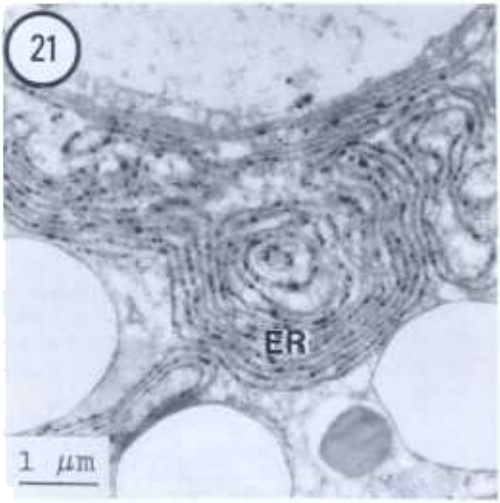
0,5 µm


21
ER
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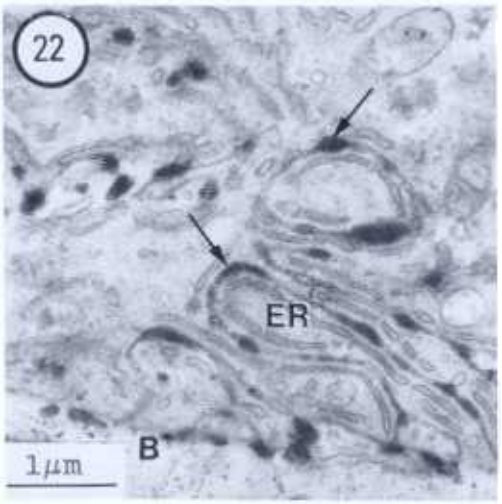
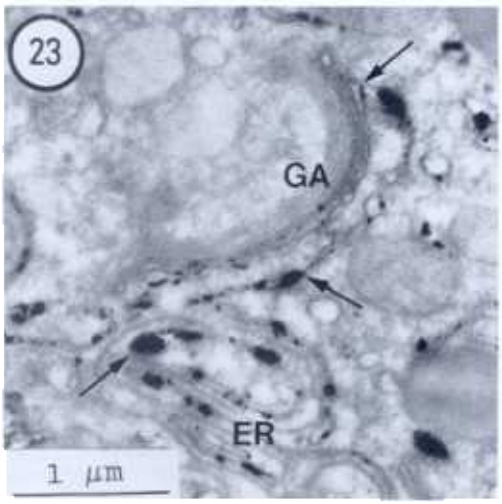
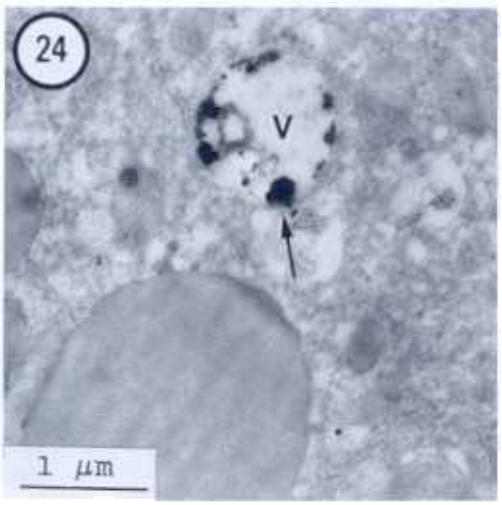

22
ER
1µm
B


23
GA
ER
1 µm


24
V
1 µm

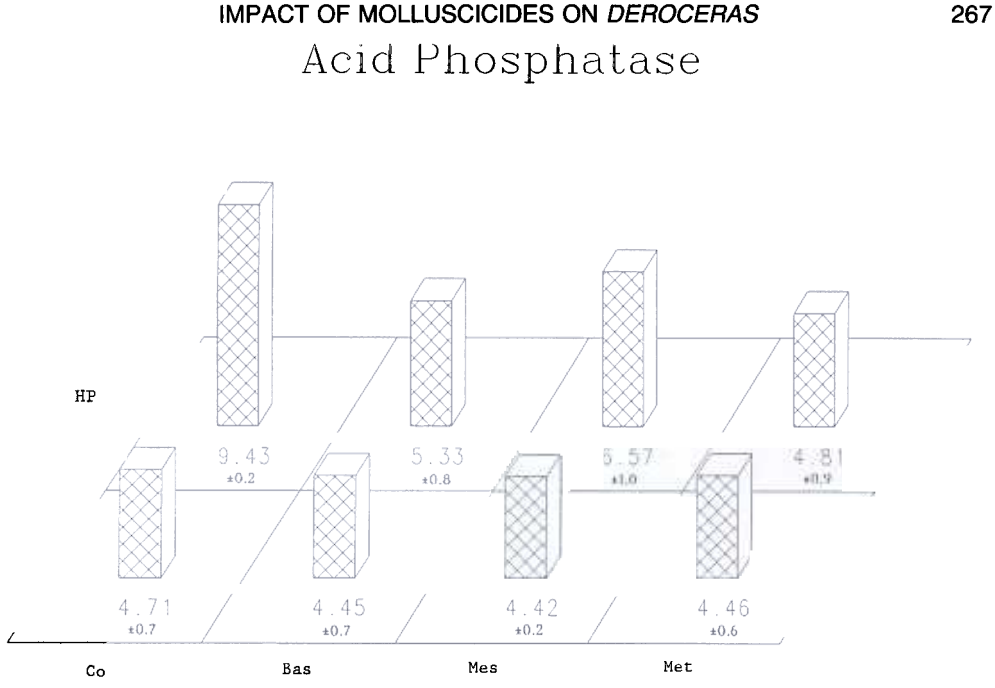


FIG. 25. Photometric measurement of acid phosphatases in the hepatopancreas (HP) and the digestive tract (DTR) of *Deroceras reticulatum* in controls (Co) and 5 h after ingestion of Cloethocarb (Bas), Mesurol (Mes) or the metaldehyde containing molluscicide Spiess Urania 2000 (Met). For each treatment, four animals were measured three times each.

the cells also supports this hypothesis. It is further supported by the results of autoradiographic studies (Triebskorn et al., 1990), in which it was demonstrated that radio-labeled Cloethocarb can be found in the crypt cells only 1 h after the first ingestion of the molluscicide.

The enhanced activity of alkaline phosphatase in the cisternae of the granular endoplasmic reticulum in the crypt cells might be due to an activation of intracellular, energy-consuming processes. It is possible either that the synthesis of special enzymes eventually involved in detoxification is activated or that intracellular transport of essential metabolites is reinforced due to an increased energy demand. The latter is supported by the fact that immediately after poisoning with metaldehyde or Cloethocarb, storage products in the cells, especially in the crop, are reduced (Triebskorn, 1989; Triebskorn & Künast, 1990), and that this reduction is proceeded by

FIGS. 19–24. Electron microscope staining of acid (AcP) and alkaline (AlP) phosphatases.
19. AcP, 2% Cloethocarb, 1 h. Enzyme-active vesicles (V) in a crypt cell arising from a Golgi apparatus (GA), ×20000.
20. AcP, 2% Cloethocarb, 14 h. Phosphatase activity in a cisterna of the Golgi apparatus (GA) and in Golgi vesicles (V), ×42000.
21. AlP, 2% Cloethocarb, 1 h. Phosphatase activity in the endoplasmic reticulum (ER) of a crypt cell, ×10900.
22. AlP, 2% Cloethocarb, 1 h. Endoplasmic reticulum (ER) in the basal part of a crypt cell (B) with enzyme activity, ×14200.
23. AlP, 2% Cloethocarb, 1 h. Crypt cell with reaction in the endoplasmic reticulum (ER) and in a cisterna of the Golgi apparatus (GA), ×19900.
24. AlP, 2% Cloethocarb, 1 h. Vesicle (V) in the basal part of a crypt cell with enzyme activity, ×15600.

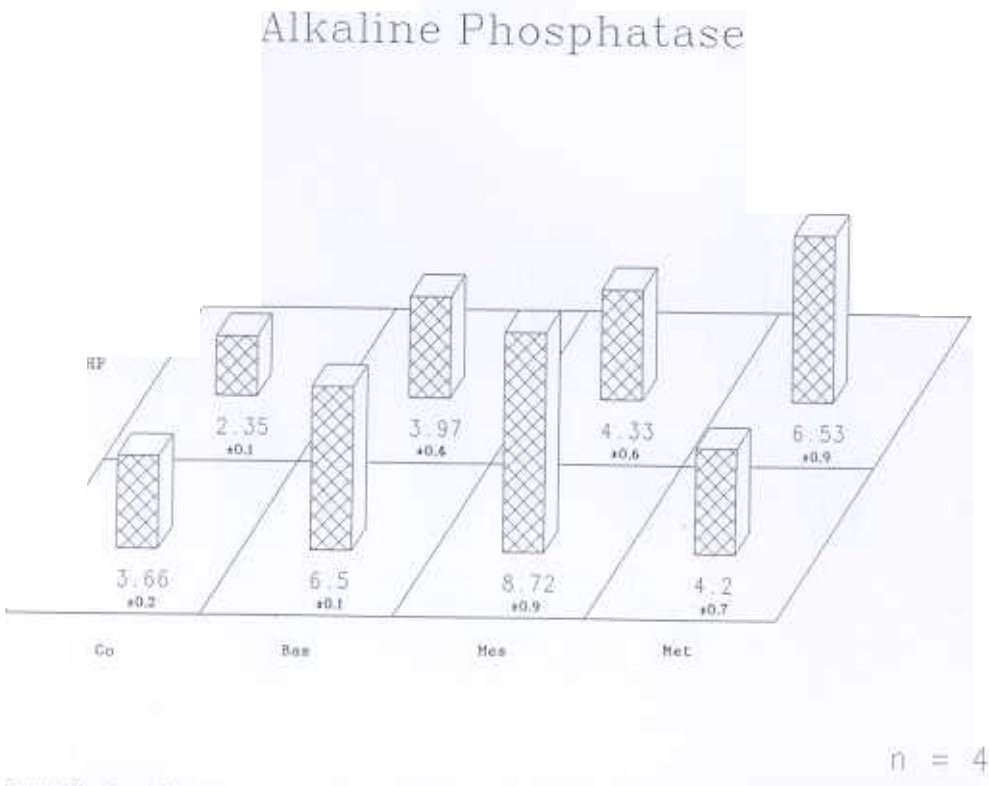


FIG. 26. Photometric measurement of alkaline phosphatases in the hepatopancreas (HP) and the digestive tract (DTR) of *Deroceras reticulatum* in controls (Co) and 5 h after ingestion of Cloethocarb (Bas), Mesurol (Mes) or the metaldehyde containing molluscicide Spiess Urania 2000 (Met). For each treatment, four animals were measured three times each.

a proliferation of the endoplasmic reticulum, which shows high alkaline phosphatase activity.

As enzymes that might be active in oxidative detoxification, NADPH-neotetrazolium-reductase (NTR) and arylhydrocarbonhydroxylase (AHH) were tested. NTR, a NADPH-cytochrome P-450-reductase with tetrazolium-salt as an artificial substrate, was used by Widdows et al. (1981), Moore & Lowe (1985), and Nott et al. (1985) as an indicator enzyme for environmental stress. Both the NTR and the AHH belong to the MFO-system (mixed function oxygenases), which is normally involved in the metabolism of steroid hormones (Lee, 1981). Enzymes of this system are characterized by a low substrate specificity (Netter, 1980). They are therefore able to bind several kinds of toxicants or xenobiotics. Furthermore, in mammals, enzymes of the MFO-system are known to be induced within a few hours (Kagan, 1988).

NTR was activated by both molluscicides tested, especially in the crypt cells. The activation after sublethal concentrations of Cloethocarb and after metaldehyde application was stronger than after application of highly concentrated Cloethocarb pellets. Because metaldehyde and low concentrations of Cloethocarb induced less degenerative effects in the cells of the hepatopancreas, but conspicious alterations of the endoplasmic reticulum (Triebskorn, 1989), two conclusions can be drawn:

First, because after metaldehyde as well as after low Cloethocarb concentrations only moderate degenerative effects occur in the cells, reactive processes may be switched on, allowing the animals to react to the poisoning. This partly agrees with the quantitation of the AHH, which is most intensely activated by met-

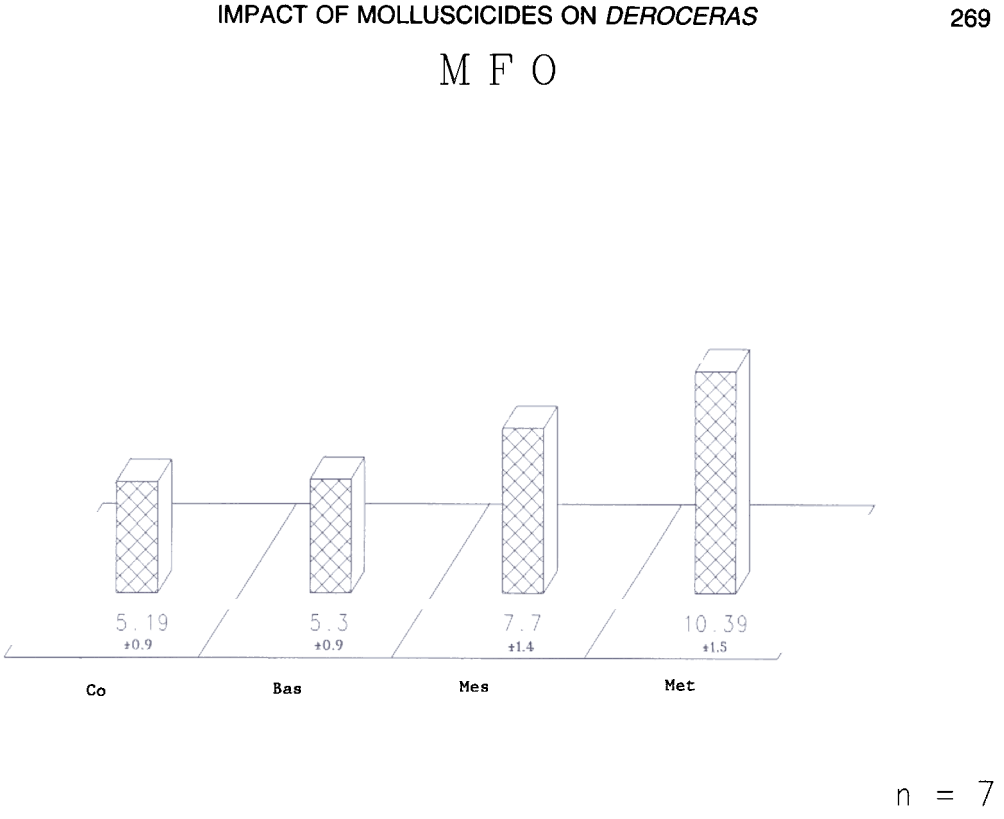


FIG. 27. Photometric measurement of the MFO enzyme arylhydrocarbonhydroxylase in control animals (Co) of *Deroceras reticulatum* and 5 h after ingestion of Cloethocarb (Bas), Mesurol (Mes) or the metaldehyde containing molluscicide Spiess Urania 2000 (Met). For each treatment, seven animals were measured three times each.

aldehyde. The reason why Cloethocarb does not induce this enzyme, whereas Mesurol, the molluscicide that leads to heavy ultrastructure damage does is not known. It is possibly due to the halogenisation of Cloethocarb.

Second, ultrastructural alterations in the endoplasmic reticulum in the crypt cells (Triebskorn, 1989; Triebskorn & Künast, 1990) may possibly be related to the activation of the NTR, which is known to be localized on the smooth endoplasmic reticulum (Smuckler & Arcasoy, 1969; Netter, 1980; Lee, 1981). The relation between changes in the endoplasmic reticulum and induction of MFO enzymes has already been described for vertebrates (Klaunig et al., 1979).

Comparing these results to those obtained in experiments with radio-labeled material (Triebskorn et al., 1990), another conclusion is that Cloethocarb is transported from the hemolymph into the crypt cells of the hepatopancreas, where metabolic processes may take place. In contrast to heavy metals, which are detoxified in the crypt cells by binding to spherites and are finally stored in these cells (Simkiss & Mason, 1984), the molluscicide is retransported after a possible metabolisation into the hemolymph. Radioactive material can be detected in hemolymph cells (Triebskorn et al., 1990).

Finally, it should be emphasized that enzymes of the MFO system are involved in the metabolism of steroids in untreated animals (den Besten et al., 1990). Only in case of poisoning, they have a function in detoxification.

Summarizing these results, it seems evident that in the digestive cells, intravacuolar digestive processes are intensified, leading to large secondary lysosomes with fragile membranes. The cells activate intracellular digestion, winning energy for reactive, energy consuming, and possibly detoxification processes that probably take place in the crypt cells. Reactions in the excretory cells are dif-

ficult to interpret because there is only little information about their function in the hepatopancreas of untreated animals. In any case, the high activity of alkaline phosphatase on the microvilli and in the large central vacuole suggests that active transport processes also take place in these cells.

In general, the question arises about the way in which enzyme activities are altered in a time span of only a few hours.

Inhibitory effects might be related to the fact that the effective substances may interact directly with enzymes as competitive inhibitors. This is often described for carbamates and cholinesterases (Young & Wilkins, 1989). Furthermore, the production of metabolites acting as inhibitors may be intensified by poisoning. Finally, the destruction of the organelles as sites of the localization of enzymes may lead to a reduction in the activity of those enzymes.

The reason for enzyme activation might be due to an interaction of the effective substance or of its metabolites with repressors, leading to the transcription of normally repressed genes. For this, the presence of inductive enzymes must be postulated. Furthermore, it is also possible that the direct interaction of the chemical or its metabolite with the respective enzyme leads to an alteration in the enzyme configuration. As a result, an enzyme with a moderate activity on the substrate used could become less specific and its activity might be increased.

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