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Copper Treatment of the Digestive Gland of the Slug *Arion ater* L. 2. Morphometrics and Histophysiology

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In order to have a clear image of processes involved in copper bioaccumulation-detoxication mechanisms in the terrestrial slug, Arion ater, we planned a histophysiological analysis based on morphometrics and on the variations in morphological characteristics of epithelia.

This study is the completion of the histochemical analysis (part 1 of this investigation), in which we suggest a close relationship between copper and calcium in the digestive gland of Arion ater. We had observed that copper was exclusively stored within calcium cells and that calcium cells secretion was more intensive at higher copper dosages and at larger bioassay times. Therefore, we thought that histophysiological analysis was necessary for explaining the nature of copper bioaccumulation-detoxication mechanisms.

MATERIALS AND METHODS

A chronic toxicity bioassay was conducted (part 1 of this investigation). The digestive gland was routinely processed, and stained with Alum-carmine (Martoja and Martoja-Pierson 1970) for morphometric analysis.

In order to determine physiological activity in the studied epithelia a morphometric analysis was made: mean epithelial thickness of glandular acini was estimated using a simple method designed by authors (Marigómez et al. 1985). The number of measures to consider was calculated using a modification of the Resources Optimization Method described by Sokal and Rohlf (1979): 10 measures per acinus, 4 acini per section and 6 sections per treatment proved sufficient for correct statistical treatment. Data were first treated in a Three Levels Enchased (TLE) ANOVA, and subsequently a Bifactorial (B) ANOVA was made in order to define copper concentration-time incidence on

Table 1. Selected morphological characteristics. We obtained a dendrogram considering the same value for each criterium. Thus, complementary characteristics for one criterium had a partitive value while excludent ones had the total value of their own criterium.

FEATURES	CRITERIA	CHARACTERISTICS
LIPOFUSCINES	size	big (10-15 µm Ø) or small (5 µm Ø)
	abundance	numerous (10*) or not
	morphology	granular or not
	colour	light yellow or deep brown
	pattern	typical or not (peculiar crystallization)
DIGESTIVE CELLS LYSOSOMES	size	big (bunch shaped) or small (points*)
	abundance	homogeneous or partially distributed by the cell
	colour	yellow or brown
	location	apical•
		basal•
CELL TYPE PRESENCE	significant presence	calcium cells (20-25*)•
		excretory cells (10-15*)•
SECRETION	type	merocrine•
		apocrine•
		holocrine•
	cell type secreted	digestive cells•
		calcium cells•
		excretory cells•
EPITHELIAL MORPHOLOGY	cellular height	prismatic cells•
		cubic cells•
		allongated•
	epithelial shape	lobed•
		digged•
		chaotic•

* per 400x visual field in a standard 14 ZEISS microscope
• complementary characteristics (i.e., apical and/or basal: 1/2 + 1/2)

Table 1. (cont.). Physiological stages of epithelia in relation to morpholical characteristics. Bibliographical references: 1, Richardot and Wautier (1972); 2, Sumner (1965); 3, Sumner (1966); 4, Walker (1970); 5, Owen (1972); 6, Moya (1973); 7, Morton (1970).

PHYSIOLOGICAL STAGES	REFS.
-size increased by activity consequence	--
-amount increased by activity consequence	---
-different components or organization	1
-darkness due to crystalline organization	1
-unkown meaning	
-different stage of absorption or secretion processes	2-6
-abundance: activity; absence: inactivity	7
-different stage of absorption or secretion processes	2-6
-depending on size: absorption or secretion	2-6
-depending on size: absorption or secretion	2-6
-absence related to calcium cycle or degenerating stage	7
-abundance after activity or before epithelial renovation	7

-secreting activity	5,7
-epithelial renovation	7
-extracellular or post-intracellular digestion	2-4
-calcium cycle or epithelial degeneration	7
-post-digestion or epithelial regeneration	2-4
-intracellular activity or absorption processes	7
-pre- or post-activity of epithelia	7
-absorption processes	7
-secretion processes	7
-epithelial regeneration or degeneration	7
-atrophic and inactive epithelia	7

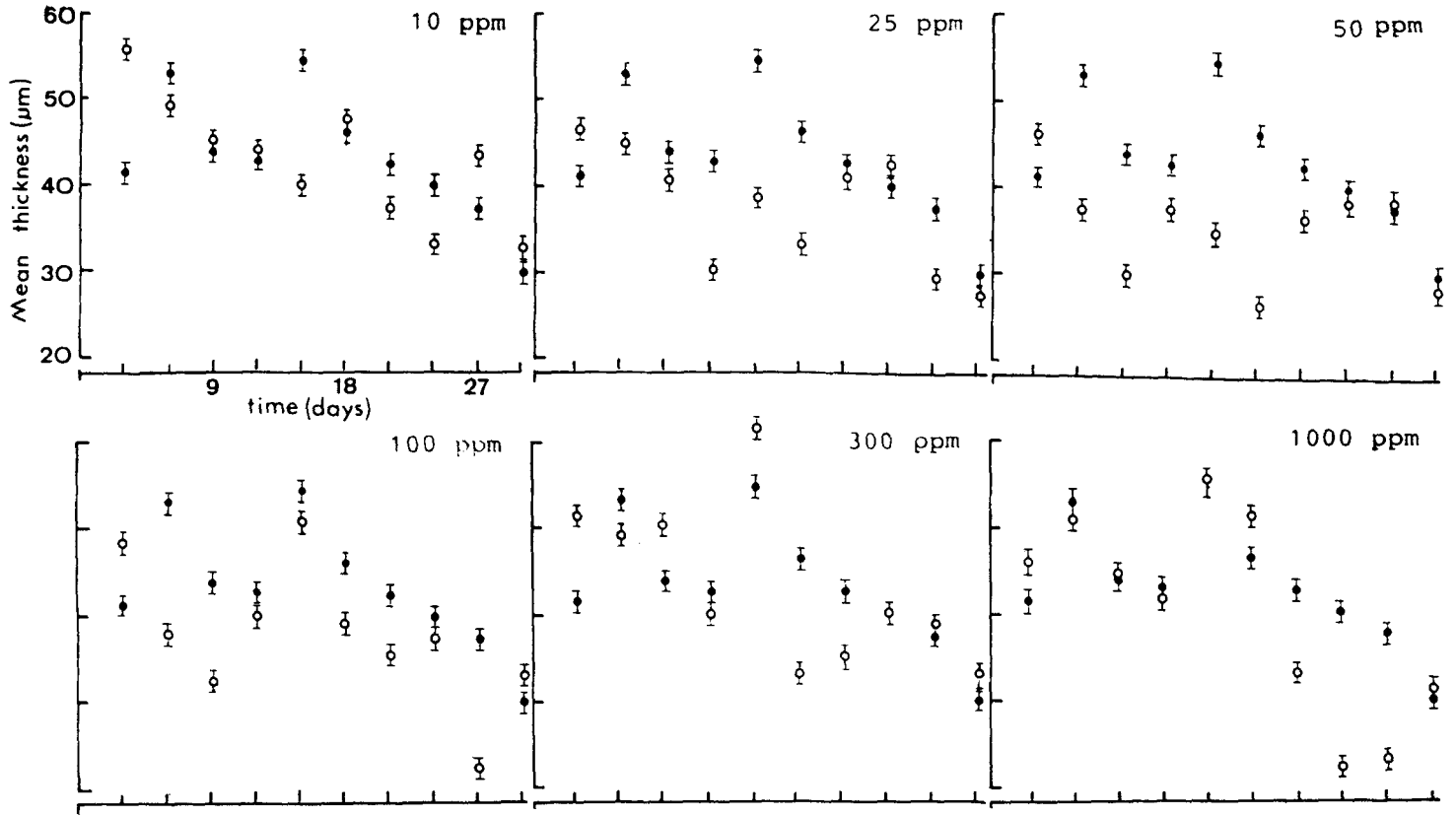

Figure 1. Results of the morphometric analysis. Dots indicate the mean epithelial thickness (µm) for control (•) and treated (o) slugs. Intervals indicate the standard 99% confidence limits.

epithelial thickness (Sokal and Rohlf 1979, Lowe et al. 1981).

We were able to correlate a description of epithelial activities with the observed behaviour in the variation cycle of epithelial thickness. After an accurate microscopical observation of the 70 studied epithelia, 23 morphological characteristics -included in 14 groups- were selected. These were related to physiological stage of epithelia: absorptive, secreting, regenerating and inactive or atrophic (Sumner 1965; 1966; Morton 1970; Walker 1970; Owen 1972; Richardot and Wautier 1972; Moya 1973; Moore et al. 1980) (Table 1). For clarity, description of epithelia includes the design of a dendrogram using a very simple index: similitude percentage among tissues (significance level: 51%).

Statistical data treatment was realized with a SANYO MBC-555 microcomputer and programmes in Turbo PASCAL (Borland Inc.) language.

RESULTS AND DISCUSSION

Measurements of the acini epithelial cells in tissue sections on control and copper treated slugs and subsequent testing of data by a TLE ANOVA indicated that the level labeled "among sections" was the best to

Table 2. Bifactorial ANOVA table: T, main effect of time; D, main effect of diet; TxD time-diet interaction; R, replication (within animals).

Source	d.f.	SS	MS	F ratio
T	9	521094.16	57899.35	202.41 (P<0.001)
D	6	146000.21	24333.37	85.07 (P<0.001)
TxD	54	502678.44	9308.86	32.54 (P<0.001)
R	16730	4785369.44	286.05	
TOTAL	16799	5955412.25		

be used as a subgroup in the B ANOVA performed to determine incidence of time and copper concentration in diet on acini epithelial thickness (there was not a significant variance component added at this level: P>0.05) (Marigómez et al., 1985). B ANOVA (Table 2) indicated that copper treatment induces a significant reduction in the mean height of epithelia (P<0.001). Time and interaction effects are also highly significant, though the incidence of interaction is lower. The intragroup mean square was used to construct the standard 99% confidence intervals for the mean

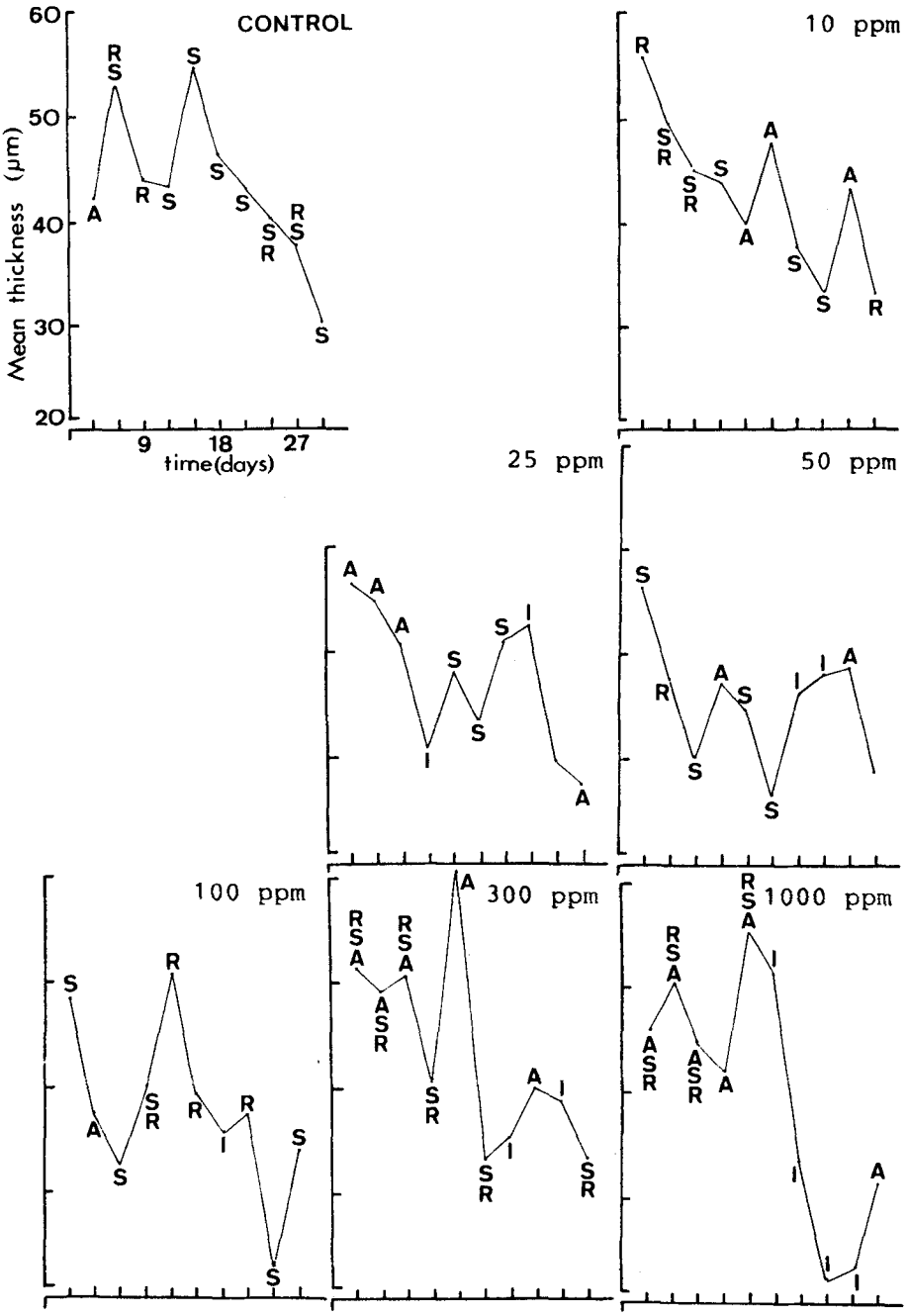

Figure 2. Relationship between main physiological stage and mean epithelial thickness of acini. Physiological stages are indicated on "mean epithelial thickness (µm) vs. bioassay time" graphics: (A) absorptive; (S) secreting; (R) regenerating; and (I) inactive or atrophic.

epithelial height for the 70 treatment groups (Lowe et al. 1981) (Figure 1).

Lowe et al. (1981) obtained similar results in mussels exposed to oil (WAF). Epithelial height reduction of a variety of tissues is usually related to stress responses in molluscs (Moore et al. 1980; Couch 1984).

Different stages of physiological activity in the various epithelia were defined in order to interpret morphometric results, namely: (a) absorptive--regenerating; (b) secreting-absorptive; (c) early secreting-absorptive; (d) late secreting-absorptive; (e) regenerating; (f) absorptive-secreting-atrophic; and (g) inactive and atrophic. Upon description of the 70 studied epithelia (facilitated by the design of a dendrogram), physiological activities were related to height variation dynamics in tissue cells. In both cases acceleration and shortenning processes took place. That can be observed in animals treated with high copper dosages (300 and 1000 ppm Cu in diet), in which physiological activity is enhanced during the first bioassay days, and subsequently the epithelium becames inactive or atrophic since the 15-18th day. Another important aspect is that regenerating activity becomes more intense in slugs treated with high copper dosages, while epithelial activity is heavily depressed. This does not occur in control and in 10 ppm Cu treated animals (Figure 2).

In the view of the results obtained in both parts of this investigation as a whole, we propose the existence of an oscillatory mechanism for copper bioaccumulation--detoxication in the digestive gland of A. ater fed on copper. Several authors (Moya 1973; Coughtrey and Martin 1976) have demonstrated the existence of endocytosis mechanisms in calcium cells of molluscan digestive gland, which can be related to the uptake of metal ions and other substances. Copper could penetrate apically by endocytosis into calcium cells, to be posteriorly accumulated in the calcium spherules until it becomes concentrated as a big granule (10-12 μm Ø) in the supranuclear portion of the cell. Copper detoxication could be acomplished by apo-holocrine secretion processes of the calcium cells. This could justify the observed parallelism between copper and calcium variation cycles, as well as the epithelial thickness reduction dependent of the copper concentration in diet. In the final step the secretion would be eliminated into faeces. Acceleration of physiological activity could facilitate this detoxication mechanism and also would be responsible for the epithelial height reduction observed in chronic experimental treatment with copper.

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