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Automated Measurement of Lysosomal Structure Alterations in Oocytes of Mussels Exposed to Petroleum Hydrocarbons

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Abstract. The present study examines the structure of the lysosomal system of mature oocytes in mussels, *Mytilus galloprovincialis*, after a 21 day exposure to the water accommodated fraction (WAF) of two crude oils (types Ural and Maya) and of a commercial lubricant oil. The automated image analysis indicates that lysosomes, showing cytochemically demonstrable β-glucuronidase activity, are smaller and much more numerous in oocytes of mussels treated with a 40% dose of Ural- and Lubricant-WAF when compared to controls. It is suggested that the structure of the lysosomal system of oocytes is different from that of somatic cells (*i.e.*, digestive cells) and that budding or "fission" into smaller bodies occurs in oocyte lysosomes under certain petroleum hydrocarbon-exposure conditions. These changes in the lysosomal compartment appear to be associated to the process of gamete release or spawning.

Alterations of reproductive functions and embryonic development are very important sublethal responses to pollution because these effects concern the single individual and very often the entire population. Evidences of hemocytic infiltration and subsequent breakdown of mature oocytes have been reported in the gastropod *Littorina littorea* exposed to the aromatic hydrocarbon 1-naphthol (Cajaraville *et al.* 1990a). Berthou *et al.* (1987) have also demonstrated severe gonadal alterations in oysters during the first two years after the Amoco Cadiz oil spill. Renzoni (1975) has shown that the water-soluble fraction of three oils was highly toxic to gametes, embryos, and larvae of *Crassostrea virginica* and *Mulinia lateralis*. Besten *et al.* (1989) have recently reported that oocytes from sea stars exposed to Cd or polychlorinated biphenyls (PCBs) for 5 months are normally fertilized but show high prevalences of abnormal embryonic development.

Although organic xenobiotics, such as PCBs, accumulate within the gonads of marine invertebrates (Hummel *et al.* 1989), the mechanisms leading to gamete damage are unknown. Lowe and Pipe (1986) found a high level of gamete atresia in mussels exposed to petroleum hydrocarbons and postulate that this is brought about by the cytotoxic effects of hydrocarbons on yolk granule lysosomes. Further, the hypothetical lysosome-mediated degeneration of gametes and the subsequent resorption of gamete material may serve to replenish the connective tissue storage cells under certain conditions (Lowe and Pipe 1987). Although detailed ultrastructural works on the evolution of degenerating atretic oocytes have been published (Dorange and Le Pennec 1989), no clear evidence of the involvement of lysosomal enzymes in the degeneration of oocytes has been given for bivalves (Pipe and Moore 1985). Further, Pipe (1985) found no indication of lysosome-mediated breakdown of the oocyte contents in starved mussels. In the freshwater gastropods *Lymnaea stagnalis* and *Biomphalaria glabrata*, an increase in the numbers of hydrolase-containing yolk granules seems to start the process of oocyte maturation and spawning, and strikingly, also the process of oocyte degeneration (de Jong-Brink and Geraerts 1982). Thus, the lysosomal compartment of oocytes could be an important target of the toxic action of environmental pollutants, which are known to alter lysosomes in other cell types of molluscs (Lowe *et al.* 1981; Moore 1985; 1988; Cajaraville *et al.* 1989; Marigómez *et al.* 1989). Digestive cells of molluscs seem to be particularly sensitive to different stress conditions and exposure to xenobiotics results in formation of abnormally enlarged lysosomes, believed to be autolysosomes (Lowe *et al.* 1981; Marigómez *et al.* 1989). Stereology is a valuable and sensitive tool to measure such changes in digestive cell lysosomes and, used together with adequate statistical methods, is able to relate lysosomal changes to exposure concentrations of contaminants (Lowe *et al.* 1981; Cajaraville *et al.* 1989; Marigómez *et al.* 1989).

The aim of the present work is to gain information about the mechanisms of cell injury caused by hydrocarbons on oocytes. With this purpose, lysosomal changes have been quantitatively evaluated by image analysis in mature oocytes of mussels after treatment with the water accommodated fraction (WAF) of three petroleum derived hydrocarbons (PHCs).

Materials and Methods

Experimental Procedure

Mussels, *Mytilus galloprovincialis*, were collected from Meñakoz, Biscay (43°24'N, 2°93'W) in March 1988 and transferred to the laboratory. Full details of the experimental design have been previ-

ously described (Cajaraville *et al.* 1990b). Briefly, mussels were exposed, after a 10-day acclimatization period, to the Water Accommodated Fraction (WAF) of two crude oils (types Ural and Maya) and of a commercial lubricant oil for 21 days in a thermostatized semicontinuous water flow system. Individuals (2.5–3.5 cm shell length) were exposed in two replicate series to three different doses of the WAF of each oil (0.6%, low dose-LD; 6%, intermediate dose-ID; 40%, high dose-HD). 100% of the water was replaced every 2 days and water was dosed every day. Two replicate control sets (without WAF) were also carried out. The WAFs of the oils was prepared by the methods described by Boylan and Tripp (1971) and Anderson *et al.* (1974).

Cytochemistry

Ten mussels were sampled after 21 days treatment from each experimental group. Portions of freshly excised digestive gland and mantle tissue were frozen with Bright Cryo-spray (dichlorodifluoromethane, −50/−55°C) and stored at −26°C. Samples were then placed on aluminium cryostat specimen holders and embedded in Bright Cryo-M-Bed. Sections, 9 μm, were cut in a Bright's cryostat (5030 microtome) at a cabinet temperature of −28/−30°C. Sections were then collected on glass slides brought from room temperature and stored at −70°C until required for staining.

The cytochemical reaction for β-glucuronidase was demonstrated in unfixed cryostat sections as in Moore (1976) with the following modifications: polyvinyl alcohol (Sigma-P 8136) at a 20% (W/V) concentration was used as colloid stabilizer, fast garnet GBC (Sigma-F 6504) was used as post-coupling agent and tissue sections were not preincubated (Cajaraville *et al.* 1989). Incubations were carried out at pH 4.5 and 37°C for 30 min and, after fixation, sections were counterstained with a 0.1% aqueous solution of fast green FCF (Sigma-F 7252) for 30 min.

Automated Image Analysis

Light microscopic images were acquired by a system consisting of a B&W-CDD TV camera, a HR-TV color screen, a Leitz light microscope, an AT-PC computer (40M) attached to a matrix printer, a PIP 1024 (Matrox) boardset and software developed by Filosoft S.A. (Spain). An objective lens of 100× magnification was used and the optical contrast of the red-brown lysosomes was improved by the use of a green filter. Binary images segregating lysosomes from oocyte cytoplasm were obtained by the segmentation procedure, which was manually adjusted in the first measurement of a given section to correct slight differences in staining intensity between different sections. Further measurements were automatically segmented. The total area scanned in each measurement was 3370 ± 750 μm^2 and 4 measurements were made in each section of 2–5 mussels per experimental group. The computer uses the stereoscopic images to generate the following parameters: lysosomal volume density (VD = V_L/V_C), lysosomal surface density (SD = S_L/V_C), lysosomal surface to volume ratio (S/V = S_L/V_L) and lysosomal numerical density (ND = N_L/V_C) where V = volume, S = surface, N = number, L = lysosomes, C = oocyte cytoplasm and D = density (Lowe *et al.* 1981).

Statistics

Parameters were tested, using a two-way analysis of variance in order to detect the effects of the type of toxicant and of the exposure-concentration. Significant differences between means were established at $p < 0.05$ level using the Duncan's test for multiple range comparison between pairs of means (Sokal and Rohlf 1979). Data of lysosomal VD and ND were logarithmically transformed before the statistical analyses (Lowe *et al.* 1981). The analyses were carried out in an AT personal computer with the SPSS/PC+ statistical package (SPSS Inc., Microsoft Co.).

Results

The results of the quantitative evaluation of the structure of oocyte lysosomes are shown in Figure 1. Values of lysosomal VD increased in oocytes of mussels exposed to HD-Lubricant when compared to controls, but changes were not significant at the <0.05 level (Figure 1a). The only significant changes in lysosomal SD were those of mussels exposed to HD-Lubricant (Figure 1b). Lysosomal surface to volume ratios were significantly increased in mussels treated with HD-Ural and ID-Maya (Figure 1c) while the numerical density of lysosomes was significantly elevated in mussels treated with HD-Ural and HD-Lubricant (Figure 1d). The latter is also illustrated in Figure 2.

The two-way analyses of variance indicate that changes in the structure of lysosomes were not different among the three types of pollutants tested, except for the parameter S/V in which a significant effect of the type of pollutant was evidenced (Table 1). In addition, changes in S/V and ND were dose-dependent while changes in VD and SD were not (Table 1).

Discussion

Exposure to the WAF of the Maya type crude oil caused few changes in lysosomal system structure of oocytes. Previous studies have shown that the WAF of this oil type is comparatively less toxic to mussels than Ural- and Lubricant-WAF (Cajaraville *et al.* 1990b). Treatment of mussels with Ural-WAF resulted in decreased size (increased S/V) of individual lysosomes while the numbers of lysosomes (ND) were markedly increased with respect to controls. Changes in these two parameters are of the same magnitude but of opposite effects and thus, significant changes in volume density and surface density of the lysosomal compartment were not detected. In the case of treatment with Lubricant-WAF, the increase in lysosomal ND was much more pronounced than the decrease in lysosomal size. This resulted in a very significant elevation of lysosomal surface density and to a moderate statistically not significant elevation of lysosomal volume density. In conclusion, lysosomes were smaller and much more numerous in oocytes of mature mussels treated with a 40% dose of the WAFs of the Ural type crude oil and of a commercial lubricant oil when compared to controls.

These results are clearly different from those obtained in a number of cell systems subjected to different stress conditions. Various forms of sublethal injury provoke the formation of giant secondary lysosomes in liver parenchymal cells and this is usually associated with a decrease in the number of lysosomes of normal size (Kerr 1973). The impact of pollutants in the lysosomal system of molluscan digestive cells has also been characterized by swelling or enlargement of

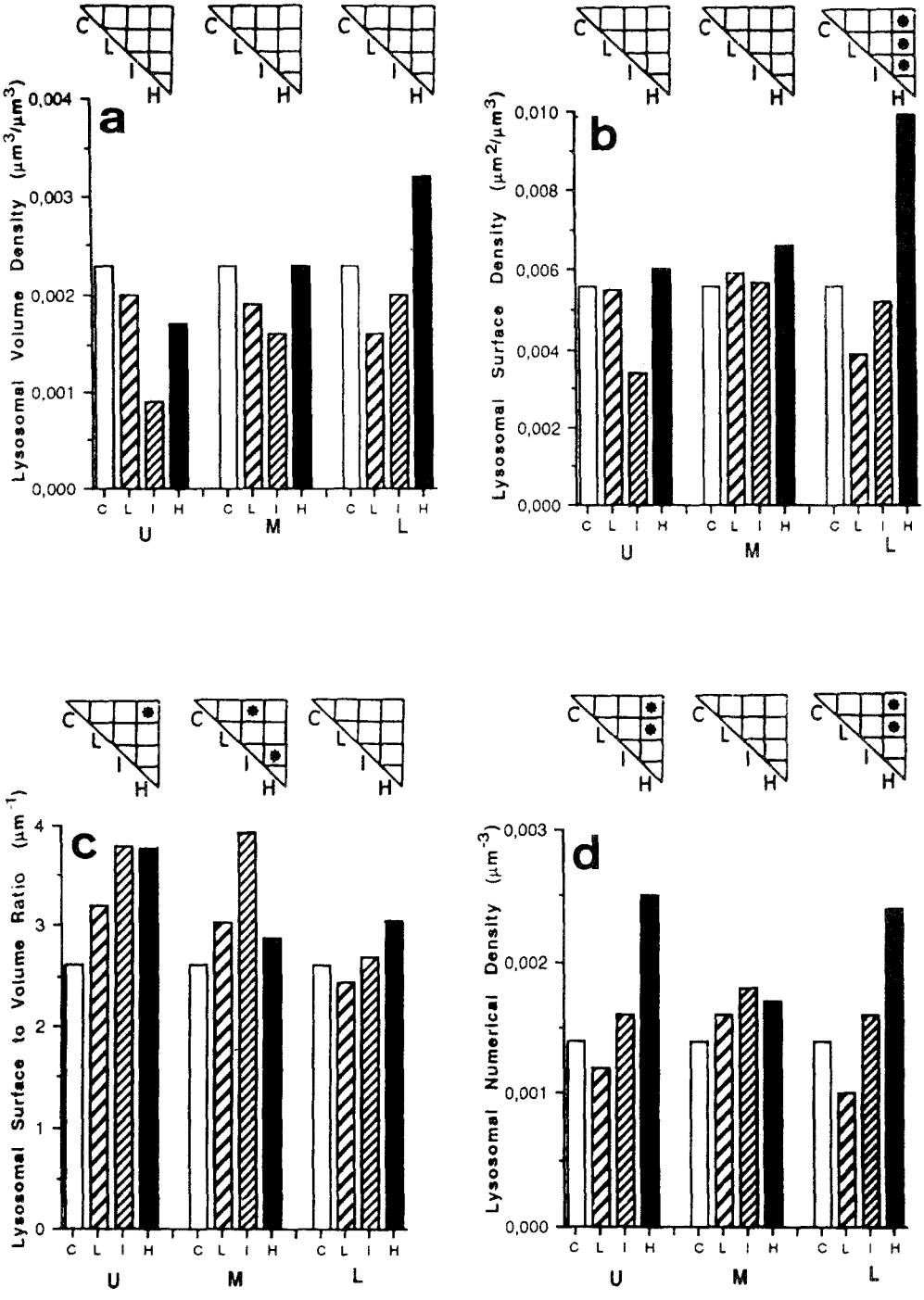

Fig. 1. Results of the stereological analysis of lysosomes in oocytes of mussels exposed to the WAF of Ural (U), Maya (M) and Lubricant (L) oils for 21 days. (a) volume density; (b) surface density; (c) surface to volume ratio; (d) numerical density. C, control series; L, low dose; I, intermediate dose; H, high dose. Significant differences between pairs of means calculated for each WAF are indicated in the upper triangular matrix by asterisks (multiple range test of Duncan, $p < 0.05$)

lysosomes, as demonstrated in some quantitative morphometric studies (Table 2). Enlargement of lysosomes has also been qualitatively determined in digestive cells of molluscs under different stress conditions (Moore *et al.* 1987; Moore 1988). Since the increased size of digestive lysosomes is usually concomitant with a reduction in their numbers, the formation of giant lysosomes (believed to be autolysosomes) appears to involve increased fusion of smaller lysosomes (Lowe *et al.* 1981). These structural changes in the digestive lysosomal system are further accompanied by a reduced stability of the lysosomal membrane, as shown by measurements of latency of different hydrolases (Moore 1985; 1988; Moore *et al.* 1987). Then, it becomes apparent that the response evoked by petroleum hydrocarbons in the lysosomal system of mussel oocytes is completely different from that induced in the lysosomal system of mussel digestive cells (Table 2) and that, contrary to the suggestions by Lowe and Pipe (1986, 1987) and Pipe and Moore (1985), the former does not appear to be associated with autophagic or degenerative processes.

As stated by Holtzman (1989), the overall volume of the lysosome population and the sizes and shapes of the individ-

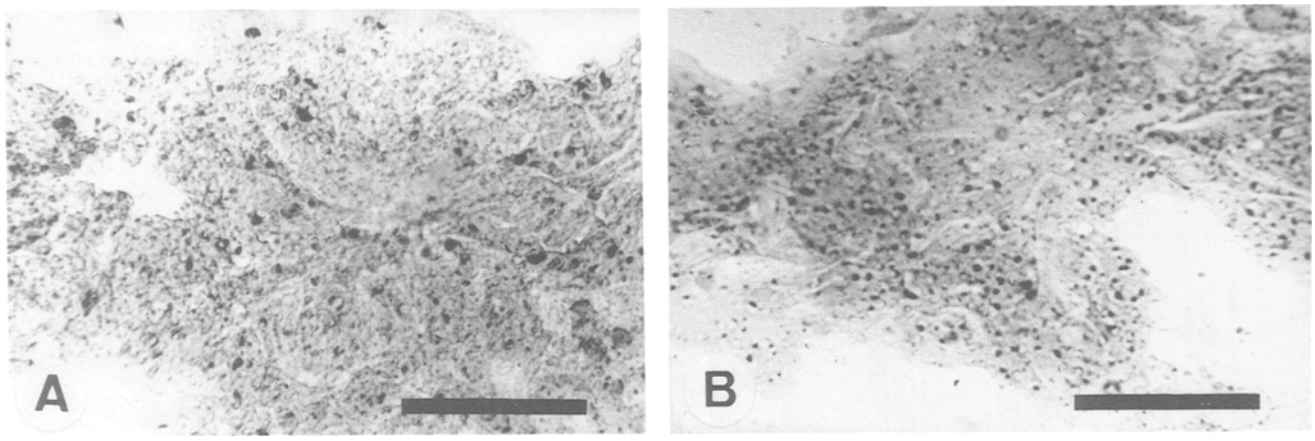

Fig. 2. Cryostat sections of the gonadal tissue of female mussels stained for the demonstration of β-glucuronidase activity. (A) Control mussel; (B) mussel exposed to the HD of Lubricant-WAF. Scale bars, 60 μm

Table 1. Summary of 2-way ANOVAs made to analyze the effect of the pollutant concentration (C) and the type of oil (T) on the structure of the lysosomal system of oocytes. VD, volume density; SD, surface density; S/V, surface to volume ratio; ND, numerical density

	F(T)[a]	df(T)	p(T)	F(C)	df(C)	p(C)	d.f.(R)
VD	1.211	3	0.319	0.611	3	0.612	37
SD	0.508	3	0.679	1.002	3	0.403	37
S/V	7.354	3	0.001	5.098	3	0.005	37
ND	1.857	3	0.154	7.015	3	0.001	37

[a] F, F ratio; P, probability of F; d.f., degrees of freedom; R, residual

ual lysosomes in given cell types depend on the existence of balances among the volume of individual lysosomes, the production of new lysosomes, the fusions of old lysosomes with one another and with new digestive structures, and the defecatory fusions with the plasma membrane. Changes in fluidity characteristics of lysosomal membranes leading to increased fusion of preexisting lysosomes may explain the effect of PHCs on molluscan digestive cells (Lowe *et al.* 1981; Moore 1985). Osmotic changes also seem to be involved in certain xenobiotic-induced digestive lysosome alterations (Cajaraville *et al.* 1989). The production of new lysosomes may be greatly diminished under conditions of exposure to PHCs, since disruption of Golgi bodies have been reported in different cell types of various invertebrates after exposure to naphthalene and to a crude oil (Robinson and Dillaman 1985; Carles *et al.* 1986; Cajaraville *et al.* 1990c). However, none of the mechanisms cited above could explain the results of the present work. It seems reasonable to suggest that the structure of the lysosomal system of oocytes is different from that of somatic cells and that some sort of budding or "fission" into smaller bodies occurs in oocyte lysosomes under certain petroleum hydrocarbon-exposure conditions. Fission of lysosomes may explain the presence of increased numbers of lysosomes, which are of smaller size, in oocytes of PHC-treated mussels.

It is interesting to note that a previous study has shown, based on gonad index values estimated through the observation of histological preparations, that gamete release is retarded under conditions of exposure to LD and ID of the WAFs, while this process seems to be accelerated in mussels exposed to the highest WAF-doses (unpublished results). Thus, alterations of the lysosomal system occur only in mussels whose spawning activity is accelerated. The association between the PHC-induced lysosomal fission and the spawning activity may be interpreted in view of the functional significance of lysosomes in mussel oocyte maturation. Several lysosomal acid hydrolases are found in both mature unfertilized oocytes and atretic oocytes (Pipe 1985; Pipe and Moore 1985). These authors believed that lysosomal hydrolases play an active role in ovarian oocyte development of mussels. Peek and Gabbot (1990) have recently shown that some lysosomal enzymes (acid phosphatase and cathepsin L) increase in activity during oogenesis, maximal activities being demonstrated prior to spawning in the spring. Hydrolytic enzymes are suggested to have a functional relationship with the normal maturation process of oocytes and may take part in yolk formation by limited proteolysis of pinocytosed protein (Peek and Gabbot 1990). Yolk constituted during vitellogenesis has been reported to disintegrate totally and to be reassembled into the definitive yolk granules in the oocytes of different molluscs (Bolognani *et al.* 1979).

In addition, the number of yolk granules reacting positively for hydrolytic enzymes in the freshwater snail *Biomphalaria glabrata* was variable depending on the degree of maturation of the oocytes (de Jong-Brink *et al.* 1976). As suggested by de Jong-Brink and Geraerts (1982), the final step in oocyte maturation seems to involve a massive increase of the number of yolk granules reacting positively for hydrolytic enzymes. Thus, taking into account the results presented here and those concerning gonad index values, it is suggested that the stress condition suffered by animals exposed to low and intermediate doses of the PHCs provoke the resorption of gonad material to meet the demands of maintenance metabolism. This event is not reflected by changes in oocyte lysosomes, since the degradation and resorption processes are mediated by hemocytes and gonoduct epithelial cells (Bayne *et al.* 1978; Pipe and Moore 1985; Pipe 1987; Cajaraville *et al.* 1990a) and also by auxiliary follicular cells (Dorange and Le Pennec 1989). On the contrary, mussels exposed to high doses of the toxicants maintain a state of partial valve closure and seem to be less impacted by the

Table 2. Quantitative changes in the lysosomal system of different molluscan cell types after treatment with different pollutants. The table gives statistically significant trends (+, increase; =, stability or nonrecognizable trends; −, decrease) observed in lysosomal VD (volume density), SD (surface density), S/V (surface to volume ratio), ND (numerical density). n.s., statistically nonsignificant trend

Cell type	Exposure	Response				Reference
		VD	SD	S/V	ND	
M. edulis digestive cells	North Sea crude oil sublethal, 103 d	+	+	−n.s.	−	Lowe *et al.* 1981
L. littorea digestive cells	1-naphthol sublethal, 4 d	+	=	−	=	Cajaraville *et al.* 1989
L. littorea digestive cells	1-naphthol lethal, 4 d	+	=	−	−n.s.	Cajaraville *et al.* 1989
L. littorea digestive cells	Cadmium sublethal, 28 d	=	−	−	−	Marigómez *et al.* 1989
M. galloprovincialis oocytes	Lubricant oil sublethal, 21 d	+n.s.	+	+n.s.	+	Present work
M. galloprovincialis oocytes	Ural crude oil sublethal, 21 d	=	=	+	+	Present work
M. galloprovincialis oocytes	Maya crude oil sublethal, 21 d	=	=	+	=	Present work

toxic action of the PHCs (unpublished results). Under such conditions, the number of lysosomes reacting positively for the β-glucuronidase test is greatly increased probably as a result of fragmentation from preexisting lysosomes. If lysosomes have an active role in oocyte maturation in mussels, as reported for other molluscs (de Jong-Brink and Geraerts 1982), it would then appear that the increased numbers of lysosomes found in mussels exposed to the HD of the PHCs reflects an increased degree of maturation of the oocytes and is a prerequisite for oocyte release or spawning.

Alternatively, PHCs could exert a direct toxic action on the cytoskeleton of oocytes and this could be further reflected as secondary changes in lysosomal structure. Fragmentation of lysosomes into smaller structures is common in cells exposed to nocodazole, which promotes the disassembly of the microtubules (Holtzman 1989). Further, the depolymerization of microtubules could also diminish the rate of fusion of the different components of the lysosomal system and may lead to the presence of increased lysosome numbers. Heuser (1989) has further demonstrated that changes in cytoplasmic pH lead to microtubule-based redistribution of lysosomes together with alterations in the fusion/fission properties of lysosomes in certain cell culture systems. But, to our knowledge, petroleum hydrocarbons have not been reported to exert a toxic action on cytoskeletal structures and further, this hypothesis does not seem feasible in view of the observations discussed above.

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