The expression of catalase in the visual system of the crab *Ucides cordatus*

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Abstract

Peroxisomes are metabolic organelles found in most eukaryotic cells and contain a variety of enzymes involved in the metabolism of reactive oxygen species, such as catalase, superoxide dismutase, and glutathione peroxidase. In invertebrates catalase has been detected in the peroxisomes of the digestive gland and in the blood cells of the mussel *Mytilus* sp, in addition to hepatopancreas and blood cells of the crab *Carcinus maenas*. The visual system, as an interface between the environment and the living organisms, can serve as a sensitive indicator of cell activities. Therefore, in this paper we studied the expression of catalase in the retina and *lamina ganglionaris* of the crab *Ucides cordatus*, since peroxisomes constitute the primary line of antioxidative defense, and in previous papers we dealt with the effects of ultraviolet radiation on the visual system of this same species. The techniques employed were immunohistochemistry, cytochemistry and immunoelectronmicroscopy. In the retina light microscopy revealed catalase in the retinular cells, and in the lamina many cells with catalase labeling dispersed around their nuclei were observed. The cytochemistry showed an electrondense reaction product on round-shaped organelles with peroxisome characteristics. These organelles were close to omocrome pigments and to other vesicles devoid of electrondense material. In the *lamina ganglionaris* organelles close to multivesicular bodies, lamellar bodies and mitochondria, and similar to those observed in the retina were observed. The immunoelectronmicroscopy confirmed that these organelles are peroxisomes. The results are discussed in terms of the involvement of peroxisomes with oxidative stress.

Key words: retina, *lamina ganglionaris*, immunohistochemistry, citochemistry, immunoelectronmicroscopy.

Introduction

Peroxisomes are single membrane-bound organelles found in the cytoplasm of most eukaryotic cells (Hruban and Recheigl, 1969; Bock *et al.*, 1980). Although the morphology of peroxisomes differs between tissues and species, they are spherical organelles surrounded by a single lipid bilayer, but also have been shown to form large reticular networks (Gorgas, 1984; Schrader *et al.*, 2000; Brown and Backer, 2003). Peroxisomal matrix proteins are synthesized on free polysomes and transported to peroxisomes (Subramani, 1993; Kurisu *et al.*, 2003).

Peroxisomes play key roles in multiple cell functions (Mannaerts and Van Veldhoven, 1993), are important in cell growth, differentiation and death (Mattes *et al.*, 1999), and especially contain a variety of enzymes involved in the metabolism of reactive oxygen species such as catalase, superoxide dismutase, and glutathione peroxidase (Singh, 1996; Orbea *et al.*, 2002; Park *et al.*, 2004). In 1966, De Duve and Baudhuin named this organelle "peroxisome" because they discovered that it oxidized certain substrates producing hydrogen peroxide (H_2O_2) by utilizing molecular oxygen (O_2). Catalase and glutathione peroxidase are two cellular defenses

that serve to remove hydrogen peroxide and avoid generation of hydroxyl radicals (Halliwell and Gutteridge, 1989).

In *Drosophila melanogaster* catalase was demonstrated in the granules of the photocytes as well as in the fat body (Hanna *et al.*, 1976; Jules *et al.,* 1990, 1991). In 1998, Schwarze *et al.* observed that the cellular oxidative defense system in flies consists predominantly of the enzymes catalase and superoxide dismutase. The catalase activity is enough to protect *Drosophila* from early mortality (Mackay and Bewley, 1989).

In other invertebrates catalase has been detected in the peroxisomes of the digestive gland and in the blood cells of the mussel *Mytilus sp* (Cajaraville *et al.*, 1992; Pipe and Livingstone, 1993), and in the hepatopancreas and in the blood cells of the crab *Carcinus maenas* (Lobo da Cunha, 1995; Bell and Smith, 1995). Including, aquatic organisms are known to contain anti-oxidant enzymes although there are marked quantitative differences among the various species (Di Giulio, 1991; Winston, 1991).

The crab *Ucides cordatus* (Linnaeus, 1763) is a decapod crustacean that lives in the mangrove forests of the Western Atlantic coast, occurring from Florida, across the Antilles, northern South America and Guyana down to the state of Santa Catarina in Brazil (Coelho and Ramos, 1972). As in other decapod crustaceans (Grassé *et al.*, 1976; Allodi *et al.*, 1995; Meyer-Rochow, 1998), the eyes of this crab are hemispherical in shape, composed of ommatidia, consisting of a cornea, a crystalline cone lens system, and a retina composed of clustered photoreceptor cells or retinular cells. The retinular cell axons end in the first neuropil called *lamina ganglionaris* or lamina. The other neuropils of the optic lobe are the external and internal medullae (Fig.1) (Grassé *et al.*, 1976; Sandeman *et al.*, 1992).

Since peroxisomes constitute the primary line of intracellular, enzymatic, and antioxidative defense, and in a previous paper we dealt with the effects of ultraviolet radiation on the visual system of the crab *Ucides cordatus* by (Miguel *et al.*, 2002), this study focused on the expression of catalase in the neurons of the retina and *lamina ganglionaris* of this same species in order to better identify these organelles. The techniques employed were immunohistochemistry, cytochemistry and immunoelectronmicroscopy.

Materials and methods

Experimental animals

All the procedures adopted for the animals use are according to the "NIH Guide and Care of Laboratory Animals" and approved by the "Use Evaluation Commission of Animals in Research of the Instituto Carlos Chagas Filho/URFJ".

Nine crabs of *Ucides cordatus* used in this study were obtained from mangroves in Itambi, Itaboraí, Rio de Janeiro, Brazil. We used adult males with carapace varying from 6.2 to 8.0 cm. The animals were maintained in holding tanks with controlled temperature (25° - 28°C) under a 12/12-light/dark cycle (natural light) and were fed to the greens base (*Rizophora mangle* and *Avicenia*) *ad libitum* until sacrifice.

Immunohistochemistry

Three animals were cryoanesthetized prior to optic stalk ablation. Optic stalks were fixed in Bouin solution, dehydrated in ethanol, cleared in xylene, infiltrated with and embedded in paraffin wax. They were sectioned at 6 mm longitudinal to the long axis of the stalks. Sections were mounted on gelatin-coated slides. The sections were treated with 10% BSA to reduce any background staining. Then they were incubated overnight with the primary antibody (Chemicon rabbit anti-catalase polyclonal) at 4° C in a moist chamber. The secondary antibody (anti-rabbit IgG CY-3 conjugate-Sigma) was then applied to the sections, which were observed under a fluorescence light microscope (Zeiss Axioskop). Controls to the reaction were prepared omitting the primary antibody.

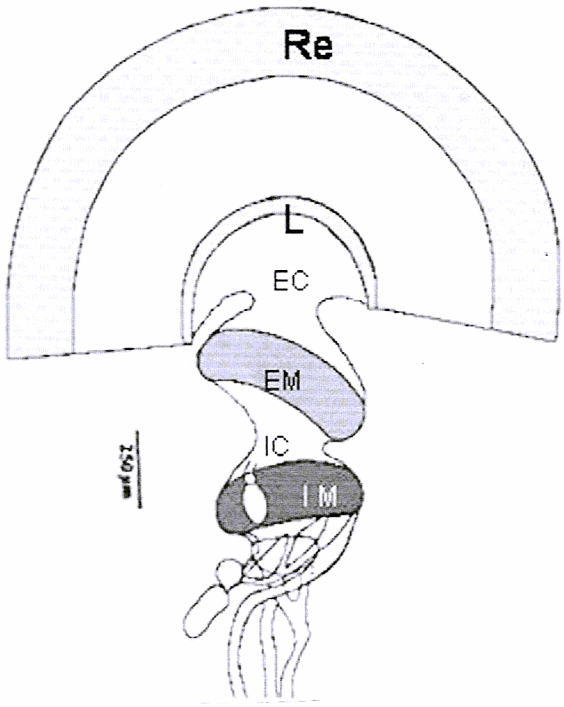

Figure 1: Schematic diagram of the visual system of decapod crustaceans. Re, retina; L, *lamina ganglionaris*; EC, external chiasm; EM, external medulla; IC, internal chiasm; IM, internal medulla. Modified from Strausfeld and Nässel (1981).

Cytochemistry

Three animals were cryoanesthetized and sacrificed, and after careful dissection, retina fragments were obtained. They were rinsed in cacodylate buffer and incubated in the 3,3'-diaminobenzidine (DAB) medium, according to the method described by Le Hir *et al.* (1979), slightly modified. Briefly, the incubation was carried out in 10 mM Teorrell-Stenhangen buffer containing 5 mM DAB and 0.15% H_2O_2 for 2 h at 37^0 in a water bath. The incubated retina fragments were post-fixed with 1% reduced osmium tetroxide and embedded in Epon. Ultrathin sections (70 nm) were stained with lead citrate for 5 min and observed under a Zeiss 900 transmission electron microscope, operated at an accelerating voltage of 80 kV.

Immunoelectronmicroscopy

Three animals were cryoanesthetized and sacrificed, and the dissected optic lobes were cut into small pieces. They were then fixed with 0.1% glutaraldehyde and 4% formaldehyde in 0.1M sodium cacodylate buffer pH 7.2 during 2 h at room temperature. The material was washed in 0.1 M sodium cacodylate buffer pH 7.2. Dehydration took place in a graded series of acetone and LR White (London Resin Company) was used as the embedding medium. Semithin sections (500 nm) stained with aqueous toluidine blue were observed under a light microscope for orientation of the sections. Ultrathin sections (70 nm) were obtained and sent to Dr. Ahmed Yagi (Oulu University – Finland), who reacted with the anti-catalase antibody and with the secondary antibody (10 nm gold-conjugated antibody) before observation under the transmission electron microscope.

Results

As stated above, the crab retina, as the seat of the photoreceptors, and the *lamina ganglionaris*, as the place of second order neurons, were the targets of our study. Fluorescence light microscopy revealed that in the retina catalase was expressed in the retinular cells close to the

rhabdoms (Fig. 2a), and that in the lamina catalase was dispersed around many cell nuclei (Fig. 2c). Most of the labeled cells presented neuron characteristics. Rat liver was used as a positive control to the reaction (not shown), and a negative control for the retina and lamina was prepared omitting the primary antibody (Figs. 2b,d).

In the retina, the cytochemical method electrondense reaction product was observed on round-shaped organelles enclosed by a membrane (Fig. 3a). These organelles, with peroxisome characteristics, were close to omocrome pigments and to other vesicles, which were devoid of electrondense material, and were scattered in the photoreceptors cytoplasm. Although we have not quantified, we could not observe an expressive number of these round-shaped organelles in the retina. In the *lamina ganglionaris* organelles similar to those observed in the retina were observed (Fig.3b). They were close to multivesicular bodies, lamellar bodies and mitochondria.

The immunoelectronmicroscopy showed gold particles on membrane-involved organelles (Fig. 3c), similar to those observed after the cytochemical reaction in the perikarya of photoreceptors, thus confirming that those round-shaped organelles are peroxisomes. Peroxisomes are present throughout the perikarya cytoplasm. The reaction was less intense but similar to that observed in the peroxisomes of the rat liver, which was used as a positive control (Fig.3d).

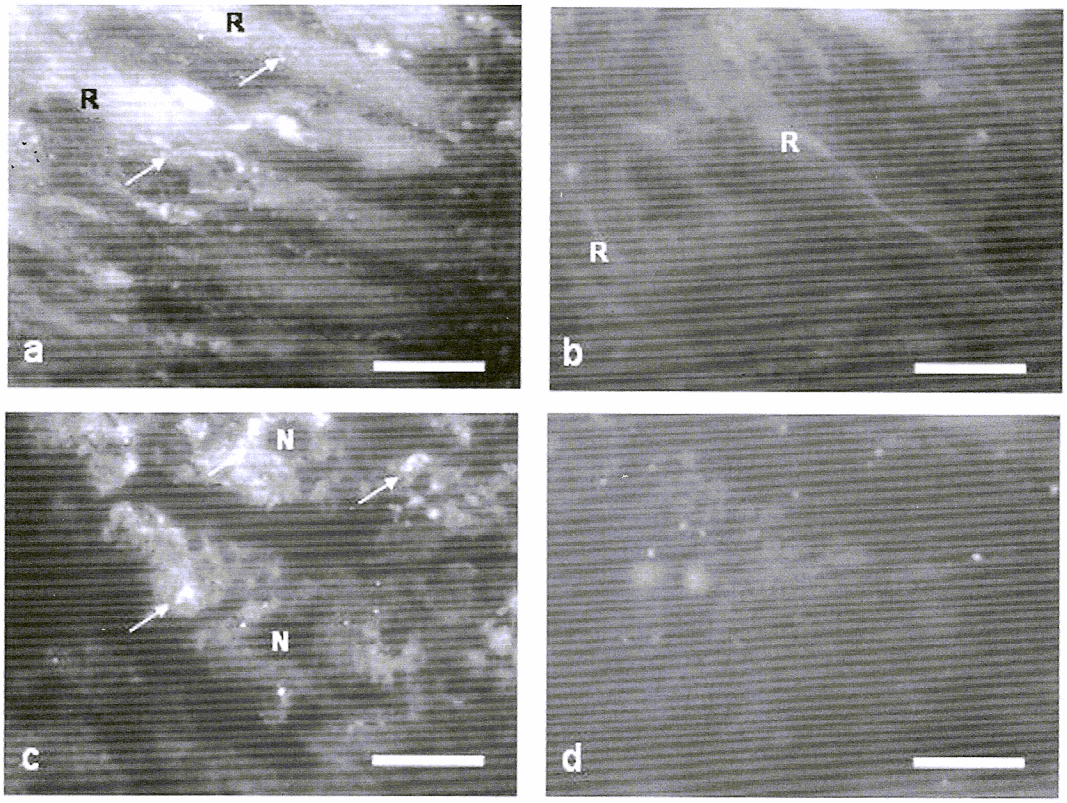

Figure 2: Catalase immunoreaction in the retina (**a**) and in the *lamina ganglionaris* (**c**) of the crab *Ucides cordatus*. Note in **a**, fluorescent structures in the retinular cells (arrows) close to the rhabdoms (R). In **c**, observe that the fluorescent reaction (arrows) is dispersed around many cell nuclei (N). **b** and **d**: Control sections near adjacent to those in **a** and **c**, respectively. In **b**, note the rhabdoms (R). Bars: 10 μm.

Discussion

Peroxisomes were first discovered by electron microscopy by Rhodin in 1954, who named them microbodies at that time, but their functions have remained obscure for many years even in the better-studied vertebrate species. De Duve and Baudhuin (1966) found that peroxisomes in the rat liver contained H_2O_2 producing oxidases together with the H_2O_2 splitting catalase and

hence, he introduced the term peroxisome for this organelle. In addition, the Cu,Zn-superoxide dismutase has been localized in peroxisomes (Chang *et al.*, 1988; Keller *et al.*, 1991) thus emphasizing the role of peroxisomes in the metabolism of free radicals. Apart from this function, liver peroxisomes in mammals are involved in several aspects of the lipid metabolism such as β-oxidation of very long chain fatty acids (Lazarow and De Duve, 1976), bile acid formation, biosynthesis of ether lipids, biosynthesis of cholesterol, precursor of steroid hormones, and catabolism of prostaglandins and leukotrienes (Osmundsen *et al.*, 1991).

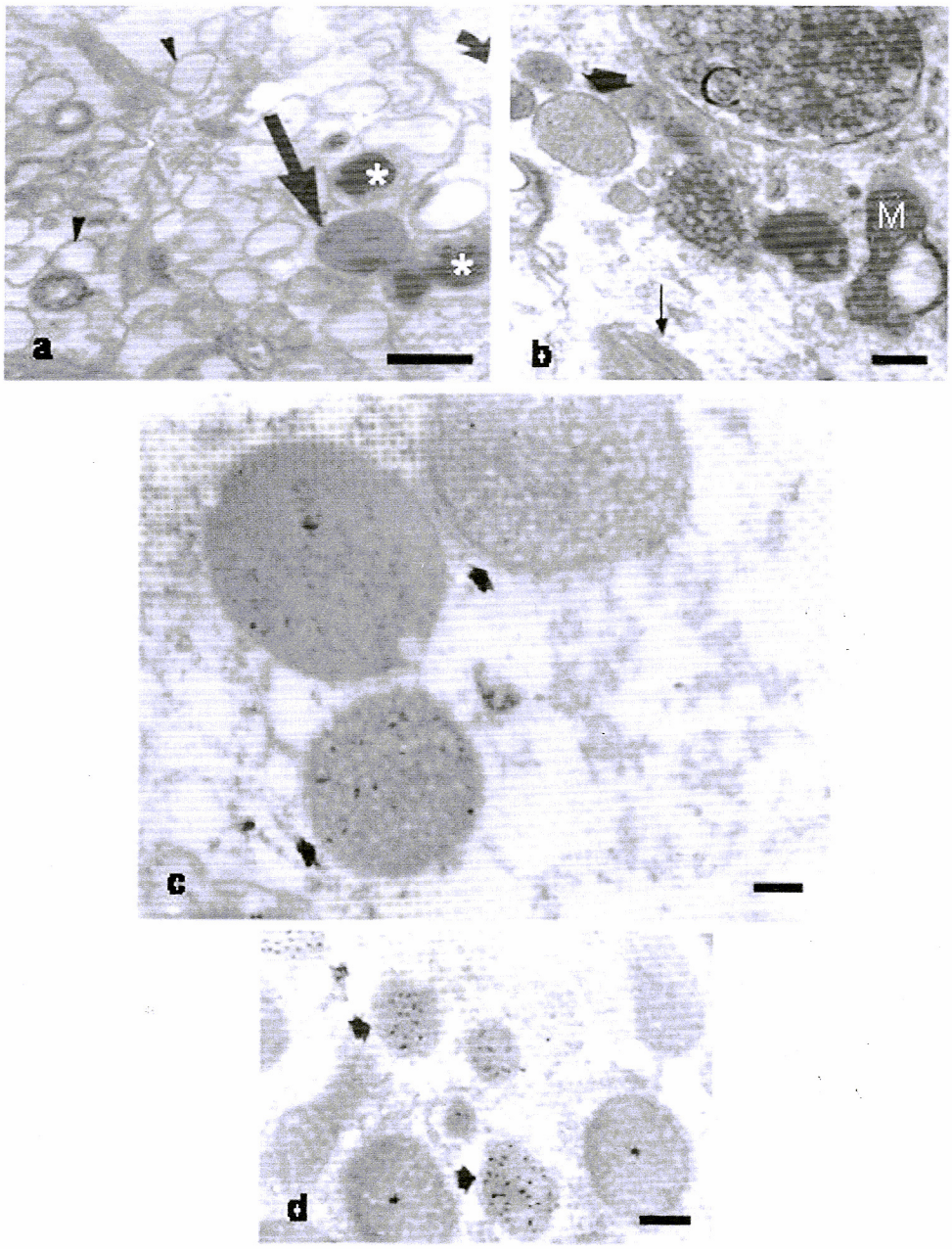

Figure 3: Catalase cytochemical reaction in a retinular cell (**a**) and in a monopolar cell in the *lamina ganglionaris* (**b**) of the crab *Ucides cordatus*. In **a** observe electrondense reaction product (arrow) on round-shaped organelles close to omocrome pigments (asterisks) and to other large and small vesicles (curved arrow and arrowheads, respectively), which are devoid of electrondense material. Bar: 1.3 μm. Note in **b** organelles similar to those observed in the retina (thick arrow) close to multivesicular bodies (C), lamellar bodies (thin arrow) and mitochondria (M). Bar: 0.6 μm. **c** and **d**: Immunoelectronmicrographs of a retinular cell (**c**) and of a rat hepatocyte (**d**) reacted with the catalase antibody. Note gold particles on membrane-involved organelles (arrows) similar to those observed after the cytochemical reaction in the perikarya of photoreceptors. Observe that the peroxisomes of the hepatocytes and those of the retinular cells are differently sized. Bars: 0.2 μm.

In our work we observed the association of peroxisomes with pigment granules. This is interesting in view of the proprieties of peroxisomes. Eyes are continuously excited by visible light and ultraviolet radiations, so they demand for high oxygen levels, which are obtained by oxidation of carbohydrates. When the demands are higher than the usual amounts, glycogen must be particularly required and that should explain also the proximity with mitochondria. When mitochondria are closely associated with peroxisomes, they can start to act more promptly and help mobilize pigment granules. The granules seem to protect cell nuclei from excessive radiation (Miguel *et al.*, 2002) and peroxisomes in addition to mitochondria could give the necessary support for them to be mobilized, thus acting on the reduction of oxidative stress. Pigment dispersion in photoreceptors was also described as a protection against oxidative damages caused by ultraviolet A and ultraviolet B radiations in the crab *Chasmagnatus granulata* by Gouveia *et al.*, (2005). Furthermore, the proximity of peroxisomes with multivesicular bodies could mean that peroxisomes are indirectly associated with Golgi complex and endoplasmic reticulum, since multivesicular bodies are commonly found in association with these two organelles (Peters *et al.*, 1991). Regarding the lamellar bodies observed close to peroxisomes, we believe that they could represent multivesicular bodies transversally sectioned.

Despite the important number of studies on peroxisome morphology and proliferation, the investigations have been almost restricted to mammals (Singh, 1996) and very little is known about the effects of environmental pollutants and radiation on the peroxisomes of invertebrates. Since in a previous paper we have observed apoptosis in the retina and lamina cells induced by ultraviolet radiation (Miguel *et al.*, 2002), this study on the same species of crustacean was conducted to help characterize peroxisomes in order to serve as a basis for future studies on interactions of crustaceans and the environment.

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References

Allodi, S; Santos, L. M. S. and da Silva, S. F. 1995. Histological study of the visual system in the decapod crustacean *Macrobrachium rosenbergii*. Brazilian Journal of Morphological Sciences, 12(1): 14-22.

Bell, K. L. and Smith, V. J. 1995. Occurrence and distribution of antioxidant anzymes in the haemolymph of the shore crab *Carcinus maenas*. Marine Biology, 123: 829-836.

Brown, L. A. and Baker, A. 2003. Peroxisome biogenesis and the role of protein import. Journal of Cellular and Molecular Medicine. 7(4): 388-400.

Cajaraville, M. P.; Völkl, A. and Fahimi, H. D. 1992. Peroxisomes in the digestive gland cells of the mussel *Mytillus galloprovincialis* Lmk. Biochemical, ultrastructural and immunocytochemical characterization. European Journal of Cell Biollogy 59: 255-264.

Chang, L. Y.; Slot, J. W.; Geuze, H.J. and Grapo, J. D. 1988. Molecular immunocytochemistry of the CuZu-superoxide dismutase in rat hepatocytes. The Journal of the Cell Biology. 107: 2169-2179.

Coelho, P. A. and Ramos, M. A. 1972. A constituição e a distribuição da fauna de decápodos do litoral leste da América do Sul, entre as latitudes 5^{0}N e 39^{0}S. Trabalhos Oceanográficos, Universidade Federal Recife, 13: 133-236.

De Duve, C. and Baudhuin, P. B. 1966. Peroxisomes (microbodies and related particles). Physiological Reviews, 46(2): 323-357.

Di Giulio, R. T. 1991. Indices of oxidative stress as biomarkers for environmental contamination. Pp 15-31. *In* Mayes M. A., Barron M. G. eds Aquatic toxicology and risk assessment, vol.14.

American Society for Testing and Materials, Philadelphia.
Gorgas, K. 1984. Peroxisomes in sebaceous glands. V. complex peroxismes in the mouse preputial gland: Serial sectioning and 3-dimensional reconstruction studies. Anatomy and Embryology, 169: 261-270.
Gouveia, G. R.; Marques, D. S.; Cruz, B. P.; Geracitano, L. A.; Nery, L. E. and Trindade, G. S. 2005. Antioxidant defenses and DNA damage induced by UV-A and UV-B radiation in the crab *Chasmagnatus granulata* (Decapoda, Brachyura). Photochemistry and Photobiology, 81(2): 398-403.
Grassé, P. P.; Poisson, R. A. and Tuzet, O. 1976. Zoología. 1. Invertebrados. Barcelona, Toray-Masson S. A., 938 p.
Halliwell B. and Gutteridge, J. M. C. 1989. Free Radicals in Biology and Medicine. Oxford: Oxford University Press.
Hanna, C. H.; Hopkins, T. A. and Buck, J. 1976. Peroxisomes of the firefly lantern. Journal of Ultrastructure Research, 57: 150-162.
Hruban, Z. and Recheigl, M. 1969. Microbodies and related particles: morphology, biochemistry and physiology. Academic Press New York, London, 296 p.
Jules, R. St.; Setlik, W.; Kennard, J. and Holtzman, E. 1990. Peroxisomes in the head of *Drosophila melanogaster*. Experimental Eye Research, 51: 607-617.
Jules, R. St.; Kennard, J.; Setlik, W. and Holtzman, E. 1991. Peroxisomal oxidation of thiazolidine carboxylates in firefly flat body, frog retina, and rat liver and kidney. European Journal of the Cell Biology, 55: 94-103
Keller, G. A.; Warner, T. G.; Steiner, K. S. and Hallewell, R. A. 1991. Cu, Zn-superoxide dismutase is a peroxisomal enzyme in human fibroblast and hepatoma cells. Proceedings of the Nacional Academy of Sciences of the United States of America, 88: 7381-7385.
Kurisu, M.; Morita, M.; Kashiwayama, Y.; Yokota, S.; Hayashi, H.; Sakai, Y.; Ohkuma, S.; Nishimura, M. and Imanaka, T. 2003. Existence of catalase-less peroxisomes in Sf21 inset cells. Biochemical and Biophysical Research Communications, 306: 169 -176.
Lazarow, P. B. and De Duve, C. 1976. A fatty acyl-coA oxidizing system in the rat liver peroxisomes: enhancement by clofibrate, a hypolipidemic drug. Proceedings of the Nacional Academy of Sciences of the United States of America, 73: 2043-2046.
Le Hir, M.; Herzog, V. and Fahimi, H. D. 1979. Cytochemical detection of catalase with 3, 3´-diaminobenzidine. A quantitative reinvestigation of the optimal conditions. Histochemistry, 64: 51-66.
Lobo da Cunha, A. 1995. The peroxisomes of the hepatopancreas in the crab *Carcinus maenas*. Journal of Submicroscopic Cytology and Pathology, 27: 427-433.
Mackay, W. J. and Bewley,G. C. 1989. The genetics of catalase in *Drosophila melanogaster*: isolation and characterization of acatalasemic mutants. Genetics, 122(3): 643-652.
Mannaerts, G. P. and Van-Veldhoven, P .P. 1993. Metabolic pathways in mammalian peroxisomes. Biochemie, 75(3-4): 147-158.
Mattes, J. M; Perez-Gomes, C. and Nunez de Castro, I. 1999. Antioxidant enzymes and human diseases. Clinical Biochemistry, 32(8): 595-603.
Meyer-Rochow, V.B. 1998. Compound eye structure: circadian rhythmicity and responses to illumination and obscurity. Pp. 102-132. *In* Eguchi, E. and Tominaga, Y. eds. Atlas of Arthropod Sensory Receptors, Springer, Berlin.
Miguel, N. C. O.; Meyer-Rochow, V. B. and Allodi, S. 2002. Ultrastructural study of first and second order neurons in the visual system of the crab *Ucides cordatus* following exposure to ultraviolet radiation. Micron, 33: 627-637.
Orbea, A; Ortiz-Zarragoitia, M; Solé, M; Cíntia, P. and Cajaraville, M. P. 2002. Antioxidant enzimes and peroxisome proliferation in relation to contaminant body burdens of PAHs and PCBs in bivalve mollusks crabs and fish from the Urdaibai and Plentzia estuaries (Bay of Biscay). Aquatic Toxicology, 58: 75-98.
Osmundsen, H.; Bremer, J. and Petersen, J. L. 1991. Metabolic aspects of peroxisomal beta-oxidation. Biochimica et Biophysica Acta, 1085 (2): 141-158.
Park, S. Y.; Kim, Y. S.; Yang, D. J. and Yoo, M. A. 2004. Transcriptional regulation of the *Drosophila* catalase gene by the DRE/DREF system. Nucleic Acids Research, 32(4): 1318-1324.
Peters, A.; Palay, S.L. and Webster, H. F. 1991. The Fine Structure of the Nervous System: Neurons and their Supporting Cells. Pp. 14-69. *In* The Neuronal Cell Body. New York, Oxford University Press.

Pipe, R. K. and Livingstone, D. R. 1993. Antioxidant enzymes associated with the blood cell and haemolymph of the mussel *Mytilus galloprovincialis*. Fish Shellfish Immunology, 3: 221-233.

Rhodin, J. A. 1954. Correlation of ultrastructural organization and changed proximal convoluted tubule cells of the mouse kidney. *In* Hruban, Z. and Recheigl, M. 1969 International Review of Cell. Biology, suppl. 3 Academic Press London, New York.

Sandeman, D.; Sandeman, R.; Derby, C. and Schmidt, M. 1992. Morphology of the brain of crayfish, crabs, and spiny lobsters: A common nomenclature for homologous structures. Biological Bulletin, 183: 304-326.

Schrader, M.; King, S. J. and Stroh, T. A. 2000. Real time imaging reveals a peroxisomal reticulum in living cells. Journal of Cell Science, 113: 3663-3671.

Schwarze, S. R.; Weindruch, R. and Aiken, J. M. 1998. Oxidative stress and aging reduce COX I RNA and cytochrome oxidase activity in *Drosophila*. Free Radical Biology & Medicine, 25(6): 740-747.

Singh, I. 1996. Mammalian peroxisomes metabolism of oxygen and reactive oxygen species. Annals of the New York Academy of Sciences, 804: 612-627.

Strausfeld, N.J. and Nässel D.R. 1981. Neuroarchitectures serving compound eyes of Crustacea and Insects. Pp 1-132 *In* Autrum, H. ed. Comparative Physiology and evolution of vision in invertebrates, B: Invertebrate visual centers and behavior I, vol VII/6B, Spring-Verlag, New York.

Subramani, S. 1993. Protein import into and biogenesis of the organelle. Annual Review of Cell Biology, 9: 445-478.

Winston, G. W. 1991. Oxidants and antioxidants in aquatic animals. Comparative Biochemistry and Physiology, 100C: 173-176.

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