Dynamics of Hepatic Melanogenesis in Newts in Recovery Phase from Hypoxia

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Abstract: The liver of lower vertebrates produces considerable amounts of molecular oxygen during hypoxia, thus the return to normoxic conditions initially brings on high values of oxygen saturation (sO_2) in even venous blood. This temporary hyperoxia triggers the oxidative process of hepatic melanogenesis. In newts rendered hypoxic by forced immersion, after 90 minutes of re-oxygenation sO_2 of mixed blood drawn from the conus arteriosus reached 96±3% *versus* 84±7% in controls ($P<0.05$), whilst the percent of melanin in histological sections of the liver rose from 8.8±2.1 to 15.4±5.4% ($P<0.01$). Melanisation of the organ was caused by Kupffer cells which invaded the parenchyma from the subcapsular layer of myeloid tissue, became engorged with melanosomes, died and assumed a globular shape. After 6 hours of normoxia, sO_2 values returned to normal and the dead cells had disappeared, but the cytoplasm of the surviving Kupffer cells exhibited fragments of residual bodies, membrane-bound clusters of undigested melanosomes.

Keywords: Hypoxia, kupffer cells, liver, melanin, newt.

INTRODUCTION

We have recently demonstrated that under anoxic conditions the liver of the newt *Triturus carnifex* activates an oxygen generating mechanism that allows the animal to survive for a relatively long time, whilst maintaining normal activity levels [1]. The working model we proposed foresees that hepatic oxygenogenesis exploits DNA as its substrate and Fe^{2+} deriving from controlled lysis of red blood cells as an electron donor. In nature, this physiological mechanism must frequently come into play, since several biological and physical factors mean that oxygen is scarce in typical amphibian habitats, such as ponds and marshes, which are anything but favourable for cutaneous respiration under immersion [2,3]. Obviously, oxygen production must be deferred just as frequently, and this triggers a second compensatory mechanism leading to the formation of large accumulations of melanin pigment in the hepatic parenchyma [1]. Indeed melanin synthesis is a biochemical process that consumes large amounts of molecular oxygen [4]: on return to the air, or anyway to normoxia, hepatic melanogenesis has the specific role of swiftly eliminating both endogenous oxygen and products of haemolytic origin generated during its genesis - such as purines [5] and reactive oxygen species (ROS) [6,7].

Some authors had already succeeded in showing a significant relation between the amounts of melanin pigment in the liver and metabolic activity levels in various species of fish, amphibians and reptiles removed from their natural surroundings [8-12]. Therefore, the severe respiratory or pharmacological treatments used experimentally in the past [1,13-15] no longer seem necessary for studying the course of hepatic melanogenesis. In fact, the oxygen concentration in air-saturated water only reaches 3-4% of that of the atmosphere [16], so the treatment of simply passing from the air to forced immersion in water in ordinary rearing tanks would appear to be more than enough to induce the liver to generate oxygen.

On the basis of these considerations, samples of *Triturus carnifex* were given two hours forced immersion at 18°C to trigger hepatic oxygenogenesis. Subsequently, we transferred the newts to damp air to arrest the process and obtain a general picture of the course of post-hypoxic melanogenesis by analysing, after different time intervals, the histological characters of the liver and the haematic parameters related to respiratory exchange: partial oxygen and carbon dioxide pressures (pO_2 and pCO_2), oxygen saturation (sO_2), and pH.

MATERIALS AND METHODOLOGY

Newts of the species *Triturus carnifex* (Laurenti) caught in the outskirts of Florence were left to acclimatise for at least one month in suitable vivaria with a 12L:12D photoperiod. All specimens were regularly fed until seven days prior to their respiratory treatment; we then stopped feeding the animals so they would not contaminate the respiratory environment with their excrements during trials. None of the animals died naturally while they were in the laboratory. The experiments were performed in spring (March to June) 2008, in accordance with protocols approved by the Italian Ministry of Health (decree n° 29/2006-B).

1. Respiratory Treatment and Groups

Specimens of the newt weighing 10-13 g, acclimated at 18°C and fasted for one week, were kept free in normoxic vivaria, and used as controls, or submitted to hypoxic treatment. Respiratory treatment consisted in two hours forced

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1874-3366/09

immersion in a tank holding 20 litres of water taken from breeding tanks, which contained 6.5 ppm oxygen (air-saturated water at 18° contains approximately 9.5 ppm). A grid just under the water surface prevented the newts from coming into contact with the air and breathing with their lungs. At the end of the two hours, the animals were transferred to a container with the base covered with a carpet of damp clay granules, so that practically their whole body was exposed to air. This phase of the treatment can be defined as the "recovery phase" from hypoxia - the "rescue phase" described by Hochachka *et al.* [17], linked to profound variations in metabolic rate and physiologically far more critical, has a different meaning. The samples were removed after two different time intervals to check the main haematic parameters and histologically analyse the liver.

Thus the main experiment covers 3 groups of 8 animals (4 males and 4 females per group): the control group (CONTROL) and two groups submitted to two hours of forced immersion followed by a 90 minutes (RECOVERY 90 min) and 6 hours (RECOVERY 6 hr) period in damp air before removal. To complete the analysis of histological changes in the liver, the organ was removed from a fourth group of 8 samples given forced immersion and allowed to stay only 45 minutes in the air; however, we did not take their respiratory and haematic parameters as they were in a transition stage, and therefore irrelevant to our experimental design.

2. Collection of Data

Heart rate was observed under the microscope on double-pithed animals after the pericardium had been opened. Subsequently, blood was drawn from the arterial cone (*conus arteriosus*) of the heart using an insulin syringe, taking 3 minutes at most, following the method described in Frangioni *et al.* [1].

Blood gas analysis values - oxygen pressure (pO_2), oxygen saturation (sO_2), carbon dioxide pressure (pCO_2) and pH - were taken with a portable i-STAT instrument (i-STAT Corporation, East Windsor, NJ, USA) using G3+ cartridges.

For histological data, we used the routine blood methods (use of the Bürker haemacytometer for red blood cell count and heparinised microcapillaries for haematocrit value). Livers were fixed in Carnoy's solution prior to embedding in polystyrene for microtomy [18,19]; sections, both normal and "bleached" with the rapid method for melanin removal [20], were stained directly with carmalum [21] or after Perls' histochemical reaction for localising ferric iron [22].

The percentage of melanin areas in the liver slices was calculated by image analysis using an Apple Mac G5 (Cupertino, CA, USA). Microscopic images were acquired with a digital Canon EOS 400D camera (Canon INC, Tokyo, Japan) and analysed by Image J 1.39 version for Mac OSX software - available from <http://rsb.info.nih.gov./ij/index.html>. Analysis on each specimen was performed on ten randomly chosen areas from two different preparations.

Fragments of liver were prefixed in cold glutaraldehyde in phosphate buffer and fixed in osmium tetroxide buffered in veronal acetate for electron microscopy [23]. The specimens were embedded in Epon and spliced into semithin and ultrathin sections. Semithin sections (1-2 µm thick) were stained with borax toluidine blue 1% and examined under a light microscope to localise the pigmented cells in the liver parenchyma; ultrathin sections were stained with uranil acetate and lead citrate [23], and examined under a Jeol mod. 1010 electron microscope (Tokyo, Japan).

3. Statistical Analysis

Quantitative data obtained from the three main groups (CONTROL, RECOVERY 90 min, RECOVERY 6 hr) were compared by variance analysis (ANOVA) using orthogonal comparisons [24]. Significance was determined at $P < 0.05$. All data are expressed as mean ± SD (standard deviation).

RESULTS

Table **1** gives the quantitative data for the CONTROL, RECOVERY 90 min and RECOVERY 6 hr groups. There were no significant differences in heart rate and red blood cell values among the three groups; since spinalisation guarantees elimination of any external factors which could interfere with the activity of the cardio-circulatory system, the condition confirms the homogeneity of the population under study. However, pH, pO_2 and sO_2 values were significantly higher in the RECOVERY 90 min group compared with either the other two (see statistical data in Table **1**). This finding, confirmed by its correlation with hepatic pigmentation (Table **1**, last column; see also later), proves that immersion in water was sufficient to activate endogenous production of oxygen in the liver and that this last phenomenon continued during the first phases of recovery - as in our previous experiments of anoxia [1]. In fact, after 90 minutes of

Table 1. Means and Standard Deviations (SD) of Parameters from Groups of 8 Specimens of Newts (4 Males and 4 Females) During the Hypoxia Experiments at 18°

Groups	Heartbeat Beats/min	Ht %	RBC Count RBCx10^9/L	pH	pCO_2 mm Hg	pO_2 mm Hg	sO_2 %	Melanin %
CONTROL	47±6	29.0±3.3	117±27	7.49±0.12	11.7±3.2	16.6±4.2	84±7	8.8±2.1
RECOVERY 90m	42±6	31.7±5.5	111±13	7.60±0.07*	9.9±1.9	26.0±6.8**	96±3*	15.4±5.4**
RECOVERY 6h	52±6	34.4±3.5	115±11	7.40±0.08	6.7±0.8	12.3±4.2	63±2	4.7±3.9

* $P < 0.05$ and ** $P < 0.01$ after orthogonal comparison. - pH: $F_{1,9} = 7.34$; pCO_2: $F_{1,9} = 2.89$; pO_2: $F_{1,9} = 13.17$; sO_2: $F_{1,9} = 7.26$; melanin: $F_{1,9} = 12.14$.

CONTROL: control group; RECOVERY 90m and RECOVERY 6h: groups of specimens kept under water for 2 hours and subsequently exposed to damp air for the time stated (90 min, 6 hr).

Heartbeat: heart rate; *Ht*: haematocrit value; *RBC count*: concentration of red blood cells; *pH, pCO_2, pO_2* and *sO_2*: pH values, partial pressure of carbon dioxide and oxygen, and oxygen saturation of blood drawn from the arterial cone; *melanin*: percentage of melanic surface in histological sections of the liver.

re-oxygenation, sO_2 reached the same value as arterial blood (96±3%), in spite of the fact that blood drawn from the conus arteriosus comprises the total "output" from the single ventricle, i.e. both oxygenated blood from the lungs (through the left atrium) and venous blood from the systemic vessels (through the right atrium). After six hours in the air there was no longer any significant difference compared with the control group (Table **1**), so the endogenous oxygenogenesis mechanism appeared to be deactivated. On the other hand, two hours of hypoxia were insufficient to provoke compensatory pulmonary hyperventilation that would have significantly lowered the pCO_2 value - as on the contrary could be observed after prolonged anoxic treatment [1].

As for the percent of melanin in the histological liver sections, DS values showed that conditions can vary considerably from subject to subject even within the same group (Table **1**). However, the RECOVERY 90 min group showed a considerably higher amount of melanin than either the other two groups, with highly significant differences on orthogonal comparison ($P<0.01$). The high levels of hepatic pigment reached after 90 minutes in the air fell to values similar to those in the control group (Table **1**); obviously the pigment must be synthesised extremely rapidly during the first phases of recovery, but once it has been elaborated it does not accumulate in the liver and is eliminated just as quickly. The variability of conditions found in each group analysed thus reflects the dynamicity of this character.

From the cytological point of view, the melanic pigment in both the controls and animals kept in the air for six hours after forced immersion (RECOVERY 6 hr) was contained in irregularly shaped isolated cells (Fig. **1A**) dislocated along the hepatic sinusoids, fully described in the literature for various species of lower vertebrates since the times of Eberth [25] and generally recognised as Kupffer cells [7], although some authors prefer the term "melanomacrophages" [8,9,12,26,27]. The pigmented territories infiltrating the hepatic parenchyma in the RECOVERY 90 min group (Fig. **1B**), on the other hand, were cellular aggregates originating

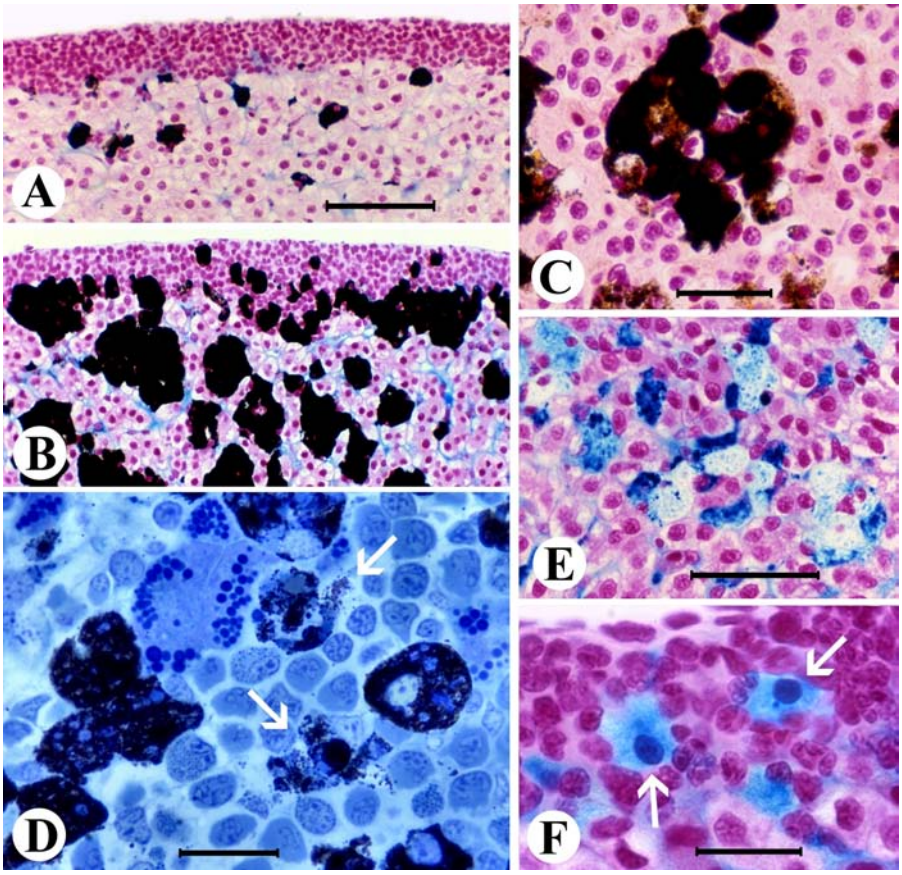

Fig. (1). Histological sections of newt liver stained with carmalum (**A,B,C,E,F**) and semi-thin section in Epon stained with toluidine blue (D). (**A**) In the controls, as in the RECOVERY 6hr group, the hepatic parenchyma is populated by individual black, irregularly shaped Kupffer cells and the subcapsular layer of the myeloid tissue that surrounds the hepatic parenchyma is homogenously thick throughout. Scale bar 100 µm. (**B**) In the RECOVERY 90 min group, pigmented cells are crowded together in large agglomerates; the subcapsular layer is hugely thickened and populated by pigmented cells that gather together and invade the parenchyma (same magnification as **A**), (**C**) The pigmented cells in the agglomerates are not homogeneous: black globular elements are closely linked with irregularly shaped yellowish-brown elements. Bar 30 µm. (**D**) 2 µm thick section in Epon at the boundary between the subcapsular layer and hepatic parenchyma: two typical Kupffer cells, irregularly shaped, with heterogeneous content and ill defined outline (arrows); on the left a small group of black globular cells, on the right an individual one. Bar 20 µm. (**E**) After bleaching out the melanin, the characteristic Prussian blue colour that Perls histochemical reaction gives the ferric catabolites shows the positive reaction of the cell population in the agglomerates with varying degrees of intensity: there is a very intense positive reaction (blue) in the originally yellowish-brown, irregularly shaped cells, whilst it is faint and widespread (pale blue) in the globular cells. Bar 50 µm. (**F**) After only 45 minutes in the air following forced immersion, Perls histochemical reaction of bleached sections shows globular cells (arrows) with deeply blue nuclei, permeated by iron. Bar 30 µm.

from the proliferation of the subcapsular myeloid layer, typical of all salamanders. In the latter group, this layer was much higher than in the controls, and its deeper portion had proliferated cells loaded with melanin that had pushed their way down into the underlying parenchyma.

The cells of the pigmented aggregates in the RECOVERY 90 min group were not morphologically homogenous: at high magnification the pigmented mass could be seen to consist of black, globular cells mixed with irregularly shaped cells, full of granules, also sometimes black, but more often between brown and ochre (Fig. **1C**). The differences in both morphology and content between the two cell types were even more obvious in slices only a few micrometers thick, such as the "semi-thin" slices preparatory for electron microscopy (Fig. **1D**) - even if the osmium used as fixative in this procedure dyes everything black so excluding the possibility to observe the natural colours of the pigments.

After histochemical removal of melanin, Perls' reaction showed that the aggregates also contained another pigment holding ferric iron, haemosiderin. Its natural colour is ochre and, in fact, it was particularly abundant and concentrated in the irregularly shaped cells (Fig. **1E**).

The histological picture was clearer when the time spent in the air after forced immersion was limited to 45 mins. In this early phase of return to normoxia, the pigmented aggregates consisted of a few cellular elements and were therefore easier to recognise. Melanin removal pointed out the difference in ferric iron content between polymorph cells, always loaded with haemosiderin, and the globular cells whose cytoplasm was often very slightly coloured but whose nuclei were intensely positive to Perls' reaction (Fig. **1F**). The association between such a marked amount of iron and chromatin is not compatible with DNA genic activity [28]: this histochemical datum is thus sufficient to state that the globular cells were dead - and actually elementary cytology tells us that the round shape of cells is already in itself a characteristic sign of the absence of activity.

Analysis under the EM documented the abundance of free melanosome distribution in the cytosol of globular cells, but recognised two different cell shapes. One was surrounded by an almost perfectly smooth plasmalemma and absolutely lacking in any other type of cytoplasmic ultrastructure (Fig. **2A**), and the other had a more irregular outline and presented large lysosomal vesicles heterogeneous in shape and density, in some of which it was possible to discern residuals of semi-digested melanosomes (Fig. **2B**). Neither of the two cell types however possessed mitochondria or any other type of cytoplasmic ultrastructure that could suggest the permanence of residual vital activities.

Fair amounts of melanosomes were also found in the polymorph cells, but their distribution was far more irregular, with characteristics corresponding more to those described in the classic descriptions in literature on Kupffer cells in lower vertebrates [7,29,30]. The cytology of these cells, however, was very rich and testified intense metabolic activity: their melanosomes could be free in the cytosol, but were more often grouped together in variously sized clusters surrounded by a membrane, mixed with further vesicles of lysosomal origin, differing not only in size and shape but also in the heterogeneous aspect of their content (Fig. **3A**, **B**, **C**).

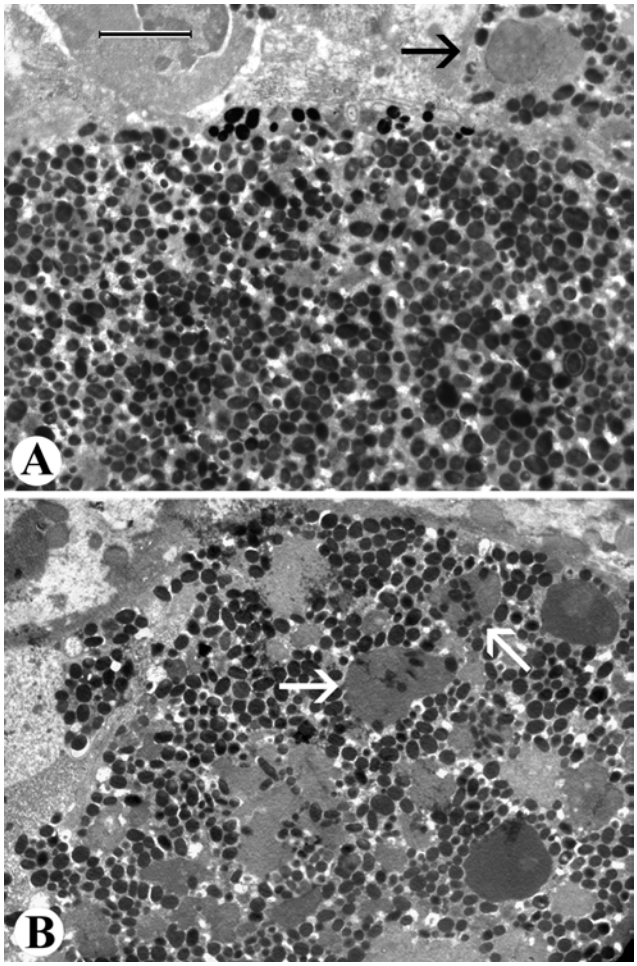

Fig. (2). Ultrastructure of globular shaped pigmented cells in the liver of a newt exposed to the air for 45 minutes after forced immersion. Scale bar 5 µm. (**A**) Large peripheral portion of a globular cell with smooth outline, with typical elliptic melanosomes of various sizes evenly distributed in the cytosol. The arrow high to the right points to a portion of an adjacent pigmented cell with heterogeneous content. **(B**) Periphery of a globular cell at an earlier stage, with more irregular outline and large vesicles of different electron-density immersed in a cytosol packed with melanosomes. Some melanosomes are also contained in a few vesicles (arrows).

DISCUSSION

Our results show that in the newt: 1) hypoxia from forced immersion is a sufficient treatment to induce post-hypoxic hyperoxia in blood drawn from the arterial cone - and thus to activate endogenous oxygenogenesis (our research hypothesis); 2) at the temperature adopted (18°C) a two hour treatment guarantees complete activation of this last process; 3) on return to normoxia, inactivation of oxygenogenesis is accompanied by hepatic melanogenesis which originates in proliferation of the subcapsular layer in the liver; 4) when melanogenesis is under way, large amounts of the melanosomes are mostly localised in dead globular cells that rapidly differentiate, and to a lesser extent in adjacent polymorph cells; 5) in the post-hypoxic period, the globular cells disappear after a few hours, but the polymorph cells, in all traits identical to the Kupffer cells of the controls, remain.

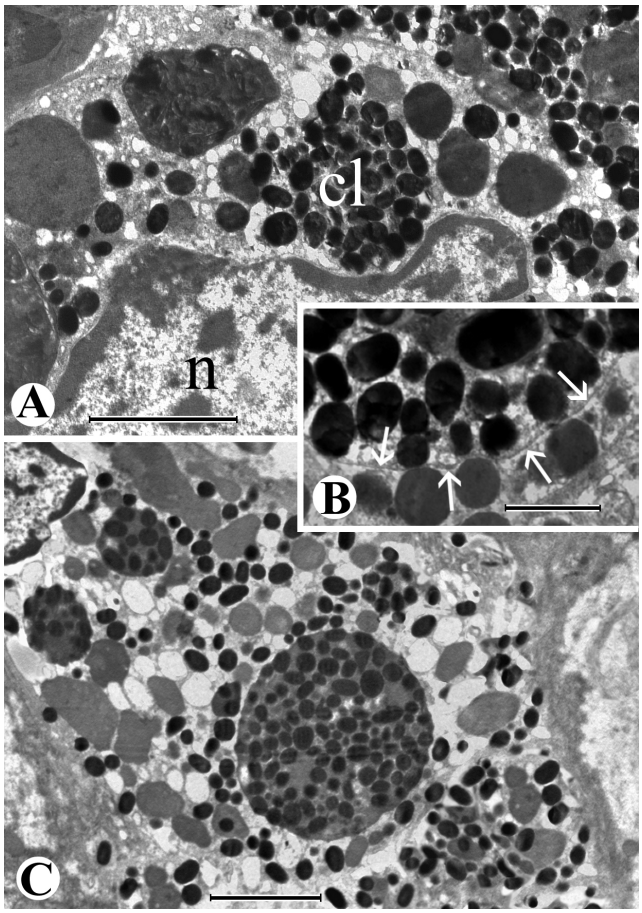

Fig. (3). Portions of stellate Kupffer cells in a newt exposed to the air for 45 minutes after forced immersion. (**A**) Next to a cluster of melanosomes (marked by the letters *cl*) the cytoplasm exhibits some free melanosomes and many vesicles of different shapes and sizes, with more or less homogeneous content and with different degrees of electron-density; *n*, nucleus. Scale bar 5 μm. (**B**) Detail of a cluster of melanosomes surrounded by a membrane (arrows) that separates them from micro-vesicles of medium electron-density. Bar 2 μm. (**C**) Vesicles, free melanosomes and, next to some other smaller ones, a large spherical cluster at a different stage of those in figures A and B, with melanosomes immersed in a substance of medium electron-density that renders the edges of the whole structure very evident. Bar 5 μm.

All these events can be coordinate to give a general picture of the response in newts to hypoxia, rather complex, but well integrated from the physiological point of view.

The Oxygenogenesis-Melanogenesis Relation

There is plenty of evidence that hepatic melanin is endogenous [7,31-36], but we also know that an ample supply of molecular oxygen is necessary for the synthesis of this pigment [4,37,38] and that because of its vascolarisation the liver is the least oxygenated organ of the entire organism [39]. On the other hand, we must also consider that the hepatic mechanism for oxygenogenesis serves to compensate hypoxia [1], so the only time when the liver can have such a large amount of oxygen available to sustain melanogenesis is when the animal returns to a normoxic environment after hypoxic crisis and temporarily "benefits" from oxygen supplies from both the outside world and its own endogenous production. This explains the high values of pO_2 and sO_2 measured in the mixed blood taken from arterial cone and the transitory melanogenesis found in the livers of the RECOVERY 90 min group. The biochemical mechanism of oxygenogenesis appears to be so complex that it cannot be halted immediately. For this reason melanogenesis, a process that can annul superfluous endogenous oxygen, lasts long enough to leave well recognisable histological traces of its activity in the globular cells loaded with melanosomes.

Death of the Kupffer Cells

A splendid globular cell, characterised by the presence of only free melanosomes in the cytoplasm, is given a full page illustration and described as a "melanocyte" of the liver of *Batrachoseps attenuatus* in the first edition of the historical "The cell - An atlas of fine structures" by Fawcett [40]. The perfect match between the morphological characters of that cell and those we found in our globular cells is more than evident - even if the term "melanocyte" would better be left to apply specifically to the classical cells of the malpighian layer of the mammalian epidermis. But a spherical shape, the absence of organelles and a nucleus impregnated with iron all allow the globular cells to be safely recognised as dead: they are so different from the well known "stellate-shaped" Kupffer cells [41] only in consequence of *post mortem* structural degeneration. In fact, Kupffer cells are the only hepatic cells with the capacity for melanogenesis,

It remains to be explained why the Kupffer cells accumulate melanin and die so quickly after hypoxic respiratory crisis, but any explanation cannot be separated from an in-depth analysis of the impact that the process of endogenous oxygenogenesis has on the organism in relation to the change in environmental conditions.

Hypothesis on Hepatic Oxygenogesis and its Control

From abundant literature it appears that in hypoxia several organs, especially the liver, produce superoxides [42-47]; thanks to catalysis by Fe^{2+} of haemolytic origin, molecular oxygen can be continuously generated from the superoxides through the Haber-Weiss cycle [48,49] - and we have been able to demonstrate that indeed in newts under anoxia haemolysis and oxygenogenesis occur simultaneously [1]. When, after respiratory crisis, an animal return to normoxia, and thus normal oxygen levels from the external environment return to the blood circulation, the equilibrium of the reaction of endogenous production of the molecule shifts to the left, and the superoxides are no longer utilised within the system and neutralised; it follows that they attack the DNA in the cells where oxygenogenesis is taking place and kill them [28]. At first the cells defend themselves by tumultuous melanogenesis that removes very high levels of oxygen [4], but in the end give in and become spherical in shape.

It should be noted that on the basis of the model expounded above (confirmed step by step by the dynamics of the results), death of the Kupffer cells identifies these macrophages as the specific seat not only of melanogenesis but also of hepatic oxygenogenesis.

Structural Evolution of Kupffer Cells

When stellate Kupffer cells die, they assume a roundish shape and simplified cytology because the enzymes in the

lysosomes characterising them as macrophages diffuse in the cytosol and attack and digest all the structures they come across, including the proteins of the cytoskeleton that support the structure of their ramifications. In fact we have described some globular cells with less regular outlines and still easily recognisable lysosomes, therefore only just dead, and others with a perfectly smooth plasmalemma and their cytosol populated by melanosomes alone. These cytoplasmatic inclusions, on account of the chemical nature of melanin (a polyindole) of which they are made, are in fact chemically far more resistant than the other organic structures [4]; this explains why they persist as the only elements that can be recognised in the cytoplasm even when the last lysosomes have disintegrated and released their enzymes. During this phase, Kupffer cells left quiescent enter into activity to eliminate the dead cells, but as long as oxygen levels in the liver remain higher than normal they also must accumulate melanosomes in their cytosol. The fragments of the eliminated cells are easily recognisable in the clusters of melanosomes surrounded by a membrane, merely representing more or less advanced stages of digestive vacuoles, surrounded by lysosomes and any endogenous melanosomes in the cytosol, as illustrated in the Results.

After a few hours of returning to normoxia, all the "oxygen generating" cells die and are phagocyted, and the process ends. Some elements still loaded with residual bodies full of melanosomes persist, but by now are quiescent. These are the Kupffer cells in the liver of lower vertebrates in the old literature, with clusters of melanosomes described as "melanogenetic centres" [7,28,29] but, as we have seen, with a completely different biological meaning.

ACKNOWLEDGEMENTS

We wish to thank Prof. Manfredo Fanfani, of the Istituto Ricerche Cliniche Prof. Fanfani SpA, and Prof. Daniele Bani, of the Department of Human Anatomy and Histology of the University of Florence, who lent much of the equipment, material and gave the logistic support necessary to carry out this project.

SCOPE OF THE ARTICLE

Experiments on recovery after moderate hypoxia confirm the finding that generation of oxygen causes melanogenesis in the Kupffer cells of the newt liver and demonstrate that its course is closely related to the respiratory conditions of the animals.

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Received: November 17, 2008 Revised: November 28, 2008 Accepted: December 01, 2008