Oxygenogenesis, a Defence Mechanism of the Newt for Surviving Anoxic Stress

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Abstract: At 8°C specimens of *Triturus carnifex*, isolated in a respiratory chamber containing water with 1.3 ppm of oxygen, completely consumed it in little over an hour; yet, even after 48 hours of anoxia, the oxygen pressure in blood sampled from the arterial cone never fell below 30% of that recorded in normoxic controls. The permanence of oxygen in the blood, incompatible with the condition of anoxia, demonstrates that there must be a physiological mechanism that produces the molecule. The liver, on account of its mass and histological structure, is the only possible seat of this process. With the supplement of anaerobic glycolysis, the oxygen produced allows the animal to survive for long periods in totally anoxic environments, a phenomenon already observed in other lower vertebrates but until now explained by glycolysis alone. Further experimental data indicate that haemolysis and hepatic melanogenesis are essential in promoting and inactivating oxygenogenesis respectively.

Keywords: Anoxia, liver, melanin, newt, respiration.

INTRODUCTION

The constant presence of melanin in the liver of lower vertebrates is a biological phenomenon which has never found a satisfactory explanation. In reptiles, amphibians and many fishes [1], large amounts of this black pigment, typical of skin and retina, accumulate in the cytoplasm of the Kupffer cells - which some authors have therefore preferred to call "melanomacrophages" [2, 3]. If hepatic melanin came from other parts of the organism, then the phenomenon could be considered no more than a curious aspect of the catabolic phase of its metabolism. However, since the early eighties, several authors working on different species have demonstrated that the fundamental enzyme for melanogenesis, tyrosinase (EC1.14.18.1), is active in the hepatic macrophages and that the pigment is synthesised *in loco* [4-9]. It is disconcerting that while biochemists have long claimed that an ample supply of molecular oxygen is indispensable for melanin synthesis [10-12], physiologists teach us (beginning from Mall [13]) that the liver is the least oxygenated organ in vertebrates - being supplied by the mainly venous blood of the portal system.

Since neither melanogenesis in an oxygen-poor environment nor the exogenous origin of hepatic melanin can be accepted as reliable hypotheses, the only remaining alternative to explain the presence of this pigment in the liver is that the organ itself can release molecular oxygen in sufficient amounts to sustain its synthesis.

At least two further considerations solicit experimental testing of this possibility: 1) several authors have already linked the melanin found in the livers of several species of fish, amphibians and reptiles to their respiratory conditions [14-18] or at least to levels of metabolic activity depending on sex [19], age [3] or season [20, 21]; 2) In hypoxia, all the organisms must resort to the anaerobic glycolysis as a source of energy complementary to aerobic respiration [22], but the anaerobic glycolysis by itself does not give a convincing explanation of the particular resistance of these animals (and of their neurons) towards anoxia, the total lack of oxygen [23]. However, during the frequent hypoxic crises aquatic species have to face owing to the physical-chemical properties of their environment [24, 25], endogenous production of oxygen might sustain steady metabolic rate much better than glycolysis alone.

Before it can be utilised, any molecular oxygen generated within an organism must be carried by the blood stream. Thus, the quickest and easiest method for testing our work hypothesis of endogenous oxygen production is that of analysing with suitable equipment the blood oxygen content of animals kept for a sufficiently long period under anoxic conditions. Alongside our basic experiment we performed a series of chemical and histological analyses on the liver and blood, aimed at determining the physiological itinerary of this possible phenomenon.

MATERIALS AND METHODOLOGY

We kept newts of the species *Triturus carnifex* (Laurenti) under anoxic conditions in a small respiratory chamber for different lengths of time to collect the most significant histological and haematological parameters. Data were also collected from groups of animals kept in humid air after their anoxic treatment during the recovery period, the "rescue phase" of Hochachka *et al.* [22]. To avoid pharmacological interferences and to prolong the times of physiological re-

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sponse, all the experiments were performed at 8°C on non-anaesthetised animals in accordance with protocols submitted to the Florence University Committee for Animal Experimentation and approved by the Italian Ministry of Health with decree n° 29/2006-B. We chose the temperature at which newts reproduce [26], well above that where signs of metabolic depression linked to hibernation could begin to occur.

The newts were caught in the outskirts of Florence and left to acclimatise for at least one month in laboratory tanks with a 12L:12D photo-period. The tanks contained islets of perforated bricks upon which the animals could climb at will. The water was continually purified and oxygenated by a recycling pump with an active carbon filter and kept at 18°C by an aquarium unit consisting of cooling and heating elements. All the specimens were regularly fed up to 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 the spring of 2006 and 2007 (March to June).

Respiratory Treatment

Specimens of newts weighing 10-13 g were fasted for one week and acclimatised to 8°C for 48 hours. Conditioning at low temperature foresees transferring the animals into a thermostatically controlled box in small 2 L tanks; it took about 6 hours to pass from 18°C in the breeding tank to 8°C - a temperature jump that can be compared to the diurnal excursion encountered in shallows ponds in sunny areas [26]. At this temperature, the animals never left the water, even though there were pieces of brick in each tank which offered them the opportunity to do so. The specimens were used as controls or put into a small 620 mL capacity sealed respiratory chamber initially filled with water containing 1.3 ppm of oxygen (air saturated water at 8°C contains 11.9 ppm). This grade of pre-established oxygenation was obtained by vacuum pumping the chamber until all of the gas had been eliminated and then adding the appropriate amount of normoxic water. In order to overcome the obstacle of the water's extremely low oxygen diffusion coefficient [25, 27], the liquid was continuously stirred by a small magnetic anchor spinning at about 250 rpm until the oxymeter registered 0.0 ppm. The anchor was then stopped, and the animal was left in the sealed chamber under absolute anoxia.

Groups

We used ten groups of four newts (two males and two females). Five groups after sampling the blood from their arterial cone - *conus arteriosus* (Fig. **1**) - furnished a complete set of respiratory, histological and haematological data (see Results, Tables **1-3**): a group of normoxic specimens served as controls (CONTROL), three groups were subjected exclusively to the anoxic treatment for 2, 24 and 48 hours respectively (ANOXIA 2h, 24h, 48h A), and a last group following 24 h of anoxia was exposed to damp air for a further 16 hours (RESCUE 16h). A sixth group of animals was subjected to anoxia for 72 hours; they did not survive the treatment but provided histological data on the liver (ANOXIA 72h). Two further groups provided data only on blood gas pressure: the specimens of the first group underwent anoxic treatment for 24 hours and were then exposed to damp air for 6 hours (RESCUE 6h); those in the second group were kept under anoxia for 48 hours, like those of one of the previous groups, but their blood was drawn from the left atrium instead of the arterial cone (ANOXIA 48h B). Finally, two groups of animals were given anoxic treatment to monitor purely qualitative effects: one group was kept under anoxia for 2 hours and then exposed to air for 2 hours to observe any peculiar anatomical aspects of the spleen, while newts in the second group were transferred to the normal breeding tank after 24 hours of anoxia to check, from their behaviour, whether the treatment had caused any functional damage to their nervous system that was not evident from histological examination.

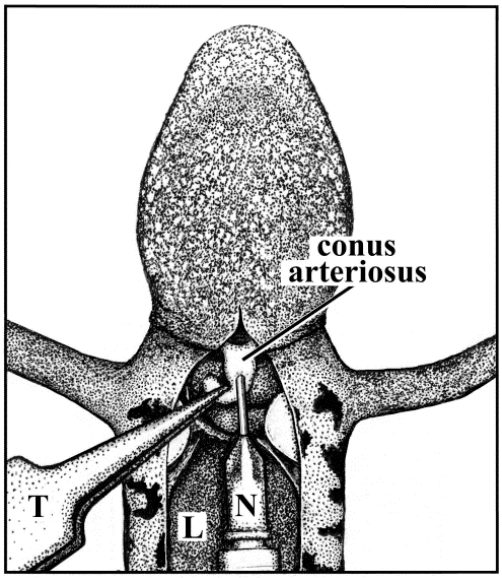

Fig. (1). Blood sample from arterial cone (*conus arteriosus*) of newt. Opening the thoraco-abdominal cavity and pericardium, with the help of tweezers (T) it is easy to insert the needle (N) of a heparinised tuberculin syringe into the arterial cone of the heart, which lies directly over the liver (L). By gently depressing the piston, the blood gradually enters into the syringe by exploiting the heart beat. The necessary amount of blood for analysis (0.25mL) was drawn within a maximum time of 5 minutes. In the case of the anoxic treatments all the operations were made on specimens submerged in paraffin oil.

Collection of Data

Oxygen concentration variations in the chamber were measured with an HI 8043 oxymeter (Hanna Instruments, Leighton Buzzard, England) and used for calculating oxygen consumption of the newts in ng/min/g.

Heart rate was observed under the microscope on the double-pithed animals after the pericardium had been opened. The blood sampling method is described in the legend of Fig. (**1**).

When sampling blood in anoxic newts, careful attention was taken to avoid any contact between the specimens and their blood with air. The pithing of the animals, the opening of the thoraco-abdominal cavity, and the drawing of their blood were all performed after each specimen had been transferred directly from the respiratory chamber to paraffin

oil at 8°C: the whole chamber was submerged in the liquid and the animal was moved in a low jar where all the procedures were carried out under paraffin. Even thin layers of paraffin oil have been proved to totally block the oxygen diffusion [28].

Blood gas analysis values - oxygen pressure (pO_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 (http//labmed.ucsf.edu/labmanual/mftlng-mtz/dnld/poct-I-STAT-1-BloodGas AndElectro.pdf). The data, given for the temperature of 37°C, were recalculated for 8°C with the formulae of Ashwood *et al.* [29]:

a) $\log(pO_2)_{8°C} = \log(pO_2)_{37°C} - (37-8)x2.303^{-1}x\ [0.058x (0.243x\ (pO_{2(37°C)}x100^{-1})^{3.88}+1)^{-1}+0.013]$;

b) $\log(pCO_2)_{8°C} = \log(pCO_2)_{37°C} - 0.019x(37-8)$;

c) $pH_{8°C} = pH_{37°C} + 0.0147x(37-8)$.

The validity of the formulae has been confirmed by comparing data supplied with the instrument software between 18° and 39°C with those obtained by calculation. The limit of instrument sensitivity at 8°C is 0.6 mm Hg for pO_2 and 6.9 for pH.

Chemical analysis of the blood plasma was carried out with a Cobas Integra 800 multiparametric analyser (Roche Diagnostics, Basel, Switzerland), using the specific kits for lactate, glucose, urea and uric acid.

Given the size of the animals and the small amount of blood that could be drawn for each blood sample (about 0.25-0.40 mL) the data for gas pressure and the chemical analysis come from different specimens.

For histological data, we used the routine methods for blood (smear technique, use of the Bürker haemacytometer for red blood cell counts and of heparinised microcapillaries for haematocrit value). Livers, brains and spleens were fixed in Carnoy's solution prior to embedding in polystyrene [30, 31]; the slices were stained with carmalum or carmalum-Azan [32]. Perls' histochemical reaction for localising ferric iron [33] was applied to spleen and liver sections, both normal and "bleached" with the rapid method for melanin removal [34].

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

Statistical Analysis

The quantitative data obtained from the various groups were compared by variance analysis (ANOVA); according to the type of comparison either one factor analysis or orthogonal comparison was used [35]. Significance was determined at $P < 0.05$. All data were expressed as mean ± SD (standard deviation).

RESULTS

In little over an hour, all the oxygen of the hypoxic chamber had been completely consumed (Fig. **2**) and the newts were left under absolute anoxic conditions. None of the newts tolerated 72 hours of anoxia, but all of them survived as long as 24-48 hours. The complex series of reactions induced by our treatments comprises four main events:

1. Genesis of Oxygen

Oxygen was found in all blood samples drawn from the arterial cone of the heart, regardless of the duration of anoxic treatment (Table **1**, ANOXIA 2h, 24h and 48h A groups). After two hours of anoxia, oxygen pressure in the blood (pO_2) had significantly fallen (from 8.8 ± 2.0 to 3.4 ± 0.6

Table 1. Means and Standard Deviations (SD) of Parameters from Groups of 4 Specimens of Newts (2 Males and 2 Females) During the Anoxia Experiments at 8°

GROUPS	Heartbeats beats/min	pO_2 mm Hg	pCO_2 mm Hg	pH
CONTROL	30±3	8.8±2.8	6.9±1.6	7.60±0.10
ANOXIA				
2h	33±5	3.4±0.6**	9.9±1.9	7.32±0.05**
24h	24±5*	3.2±0.8**	6.7±0.8	7.02±0.06**
48h A	5±2**	2.8±1.6**	5.1±0.4	<6.9[a]
48h B	/	<0.6[a]	4.7±1.2	<6.9[a]
72h	0	/	/	/
RESCUE				
6h	/	14.6±3.6*	3.8±0.4**	7.67±0.04
16h	22±3*	9.3±1.1	5.6±0.9	7.73±0.08

*P < 0.05 and ** P < 0.01 after comparison with control group.

[a]Limit of sensitivity of the i-STAT instrument at 8°C.

CONTROL: control group; ANOXIA: groups of specimens kept under anoxia for the number of hours stated (2h, 24h, 48h A and B, 72h); RESCUE: groups of specimens subjected to anoxic treatment for 24 hours and subsequently exposed to damp air for the number of hours stated (6h, 16h).

Heartbeats: heart rate; pO_2, pCO_2 and *pH*: partial pressure of oxygen and carbon dioxide, and pH values of blood drawn from conus arteriosus for all groups except for ANOXIA 48h B, where the blood was taken from the left atrium.

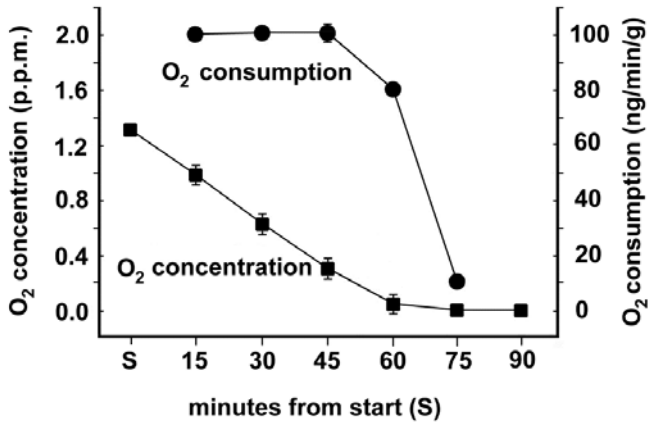

Fig. (2). Oxygen concentrations in the respiratory chamber (small squares) in parts per million (ppm), recorded every fifteen minutes during hypoxic treatment, and oxygen consumption of the newts (circles) in ng/min/g. Each point represents the mean from eight readings; the vertical bars indicate the standard deviations. S: start of experiment.

mmHg; $P < 0.01$), but after almost 4,000 heart beats was still over 38% of the initial value - too high to be considered as residual oxygen considering that cardiac muscle was perfectly efficient, heart rate did not significantly differ from that of controls nor was there any sign of general or local blood stagnation. Moreover, during the anoxic treatment, blood pO_2 remained relatively steady (3.4 ± 0.6 after 2 h, 3.2 ± 0.8 after 24 h, 2.8 ± 1.6 after 48 h; orthogonal comparison: $P > 0.05$, NS), rather than falling or decreasing in time - which implies a continuous oxygen production. From now on, this process shall be referred to as "oxygenogenesis".

The total lack of oxygen in the blood drawn from the left atrium (Table **1**, ANOXIA 48h B group) is a highly significant result because: 1) it confirms the validity of the methods adopted in order to avoid contaminating the samples; 2) it eliminates the theoretical possibility that O_2 found in the arterial cone is "residual"; 3) it proves that the molecule reaches the arterial cone of the heart *via* the systemic veins where the liver is the only organ that, on account of its mass and histological structure, can release such an amount of gas.

As further evidence of endogenous oxygenogenesis, blood pO_2 in the anoxic newts' group re-oxygenated for 6 hours (RESCUE 6h) reached a significantly higher value than in the controls (14.6 ± 3.6 vs 8.8 ± 2.8 mm Hg; $P < 0.05$). Only a process of genesis can explain an excess of oxygen in the bloodstream just when the oxygen debt should be paid back: in an aerial environment, the blood running in the respiratory organs always reaches a saturation point, both in the controls and in specimens in the rescue phase. However the latter, as a result of the oxygen debt accumulated during anoxia, consume more oxygen, so that less should remain in the mixed blood of the arterial cone. The excess must therefore be generated within the organism: in the blood of animals removed from the anoxic chamber and exposed to air, at first, there was at the same time endogenous gas as well as that coming from the external environment. The surplus was disposed of in specimens re-oxygenated for 16 hours (Table **1**, RESCUE 16h group) - in which the mechanism of oxygenogenesis had evidently been "switched off".

2. Lactic Acidosis

The decay of cardiac activity, which led at first to bradycardia and then to heart failure and death of the animals (see Table **1**, ANOXIA groups), does not appear to be caused mainly by exhaustion of the oxygen producing mechanism as by a shift in blood pH from physiological values to limits incompatible with the functions of the cell membrane - see Pinder *et al.* [36]. Pithing the animals, which frees the heart from nervous control, renders the datum of heart rate an excellent proof of muscle efficiency and consequently of the condition of the entire organism.

After the first 24 hours of anoxia, when blood pH remained over 7 (Table **1**), the animals appeared alive and viable, resumed their normal activities as soon as they were set free and their encephala appeared histologically intact. After 48 hours of treatment however, when pH level had fallen below 6.9, the blood pO_2 was still relatively high but the heart rate was too low and the nervous tissue appeared to be seriously damaged (Fig. **3**). Even the erythrocytes in circulation, which after the first 24 hours of anoxia already showed significantly higher volumes than the controls, appeared even more swollen (see red blood cell counts, haematocrit values and the resulting mean corpuscular volume in Table **2**).

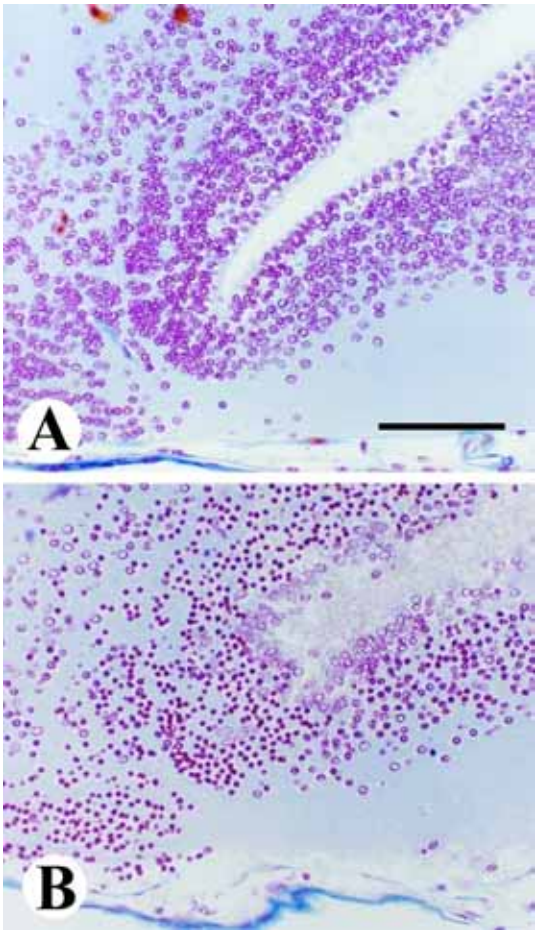

Fig. (3). Histological sections of newt encephalon stained with carmalum-Azan. Scale bar 200 μm. (**A**) Rhinencephalum from a control specimen. (**B**) After 48 hours of anoxic treatment, the same area shows obvious histological disorganization and most of the nuclei affected by pyknosis (indicating cell death).

Table 2. Means and Standard Deviations (SD) of Para-meters from Groups of 4 Specimens of Newts (2 Males and 2 Females) During the Anoxia Experiments at 8°

GROUPS	Ht %	RBC count RBCx10^9/L	MCV fL
CONTROL	23.7±3.9	78.28±14.25	3031±146
ANOXIA			
2h	32.1±3.5*	100.24±11.78	3205±252
24h	34.9±6.5*	99.69±18.86	3507±158**
48h A	28.2±2.7	68.13±12.26	4185±365**
RESCUE			
16h	21.5±2.0	79.24±3.67	2808±128

*P < 0.05 and **P < 0.01 after comparison with control group.
CONTROL: control group; ANOXIA: groups of specimens kept under anoxia for the number of hours stated (2h, 24h, 48h A); RESCUE: group of specimens subjected to anoxic treatment for 24 hours and subsequently exposed to damp air for 16h.
Ht: haematocrit value; *RBC count*: concentration of red blood cells; *MCV*: mean corpuscular volume.

Blood plasma analyses (Table **3**) revealed that lactic acid, which gradually increased in concentration during anoxia, was the cause of this acidosis, while blood pCO_2 never differed significantly from that of the controls (Table **1**). The increase in circulating lactate indicates that during anoxia insufficient oxygen was produced to entirely sustain adequate levels of metabolic activity, and that the missing quota of energy was supplied by anaerobic glycolysis - as Bennett and Licht [37] observed for some time in amphibians under less extreme respiratory conditions. After 24 hours of anoxia, blood glucose levels also had risen significantly (values and statistical comparisons in Table **3**) and remained high even when the heart rate began to slow down and the efficiency of the cardiovascular system was falling. Analyses of plasma concentrations of urea and uric acid confirm the gradual impairment of metabolic activities: after 24 hours of anoxia, a significant decrease in urea concentration corresponds to the appearance of uric acid, which after a further 24 hours reached concentrations of almost 1 mg/dL.

In animals kept under anoxia for 24 hours and then exposed to air (RESCUE groups), after 6 hours of oxygenation, pH had already reached the control value, but there was a significant drop in pCO_2 (Table **1**) probably due to pulmonary hyperventilation. After 16 hours, most of the rescue phase was completed and uric acid had disappeared from the blood plasma, while many of the haematic parameters were re-established to the values of the control group. However, heart rate and urea concentration still remained below the norm, while lactate fell from the high levels attained during anoxia to significantly lower levels than the controls (values and comparisons in Table **1** and Table **3**).

Histological analyses have allowed identification of the last two events strictly linked to the newts' response to anoxia: a diffused haemolytic process, detectable from the very first hours of treatment, and hepatic melanogenesis, a phenomenon limited to the rescue phase.

3. Anoxic Haemolysis

Anoxic haemolysis, an ample but non-catastrophic process, involved a proportion of red blood cells (RBC) roughly corresponding to the large amounts that newts can store as a reserve in their spleens [38, 39]. Thus, notwithstanding the

Table 3. Means and Standard Deviations (SD) of Parameters from Groups of 4 Specimens of Newts (2 Males and 2 Females) During the Anoxia Experiments at 8°

GROUPS	Lactate mg/dL	Glucose mg/dL	Urea mg/dL	Uric Acid mg/dL	Melanin%
CONTROL	29.8±8.5	27±10	80±18	0.00	5.1±2.3
ANOXIA					
2h	49.9±19.8	34±10	83±22	0.00	6.2±3.7
24h	122.0±13.9**	99±22**	44±8**	0.22±0.19	6.3±1.5
48h A	153.9±10.2**	80±15**	57±14	0.98±0.26	5.5±2.6
72h	/	/	/	/	5.9±2.4
RESCUE					
16h	16.6±1.8*	33±10	34±2*	0.00	19.5±8.5*

*P < 0.05 and **P < 0.01 after comparison with control group.
CONTROL: control group; ANOXIA: groups of specimens kept under anoxia for the number of hours stated (2h, 24h, 48h A, 72h); RESCUE: groups of specimens subjected to anoxic treatment for 24 hours and subsequently exposed to damp air for 16h.
Lactate, glucose, urea, uric acid: concentration of these four substances in the blood plasma; *melanin*: percentage of melanic surface in histological sections of the liver.

substantial loss of erythrocytes, the RBC counts of all the anoxic groups remained practically unchanged in respect of the controls (Table **2**); the difference between ANOXIA 2h and ANOXIA 48h group, however, results statistically significant (100.24 ± 11.78 vs 68.13 ± 12.26 RBCx10^9/L; $P < 0.01$).

Signs of haemolysis could be observed at nearly all levels. After two hours of anoxia, numerous fragments of disrupted RBC appeared in the blood smears (Fig. **4A**), "ghosts" (empty membranes) in the haematocrit capillaries formed enormous "buffy coats" (white layers) over the intact RBC and the escaped haemoglobin reddened the plasma above. In the RESCUE 16h group, there was so much iron from the haemolytic process that the Kupffer cells had accumulated large quantities of haemosiderin (Fig. **4B**, **C**, **D**, **E**) and the same blood plasma in the veins supplying the hepatic parenchyma resulted positive to the Perls' reaction (highly specific for Fe^{3+}).

A particular characteristic was the anatomical aspect that the spleen assumed when, after two hours of anoxia, the haemolytic process was precociously blocked by exposing the animals to the air. Large, white roundish bulges protruded from the surface indicating seats where residues of disrupted erythrocytes subtracted from the blood stream had accumulated (Fig. **4F**, **G**).

4. Hepatic Melanogenesis

The only significant change in the percentage of melanin in the histological sections of the liver, measured by computerised morphometry, was in the RESCUE 16h group (19.5 ± 8.5 vs 5.1 ± 2.3 % of the control group; $P < 0.025$). So much melanin had been deposited in the hepatic parenchyma of these newts that it occupied almost 1/5 of the whole liver mass (Table **3**, Fig. **4B**, **C**). It should be noted that this phenomenon manifested itself some hours after the blood pO_2 was increased to its highest value (Table **1**).

DISCUSSION

About Methodology

The respiratory chamber was initially filled with hypoxic water containing 1.3 ppm of oxygen, a precaution adopted given that the passage from normoxia to anoxia would have been too sudden a change compared to naturally occurring events in the environment. It should be noted in Fig. (**2**) that

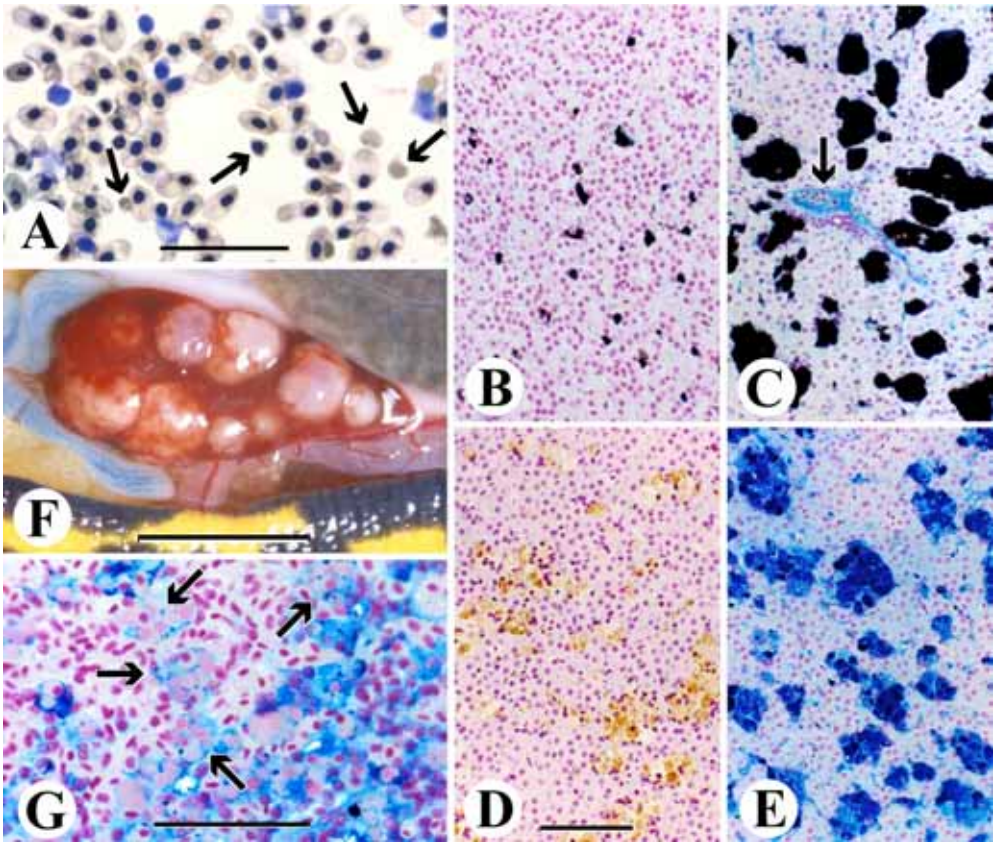

Fig. (4). Haemolytic response to anoxic treatment. (**A**) Blood smear from newt submitted to 2 hours of anoxia, stained with the May Grünwald-Giemsa method. Scale bar 100 µm. Fragments of erythrocytes (arrows) and pale blue patches of disrupted nuclei indicating that haemolysis is in progress. (B-E) Sections of newt liver stained with carmalum. Scale bar 200 µm. (**B**) Control specimen with small black Kupffer cells (Kc). (**C**) Specimen exposed for 16 hours to damp air after 24 hours of anoxia, with hypertrophy and hyperplasia of the Kc and a vessel (arrow) in which the blood plasma (pale blue) is positive to Perls' reaction. (**D**) Removal of the melanin shows the natural ochre-yellow colour of the haemosiderin in the same Kc which are stained blue in (**E**) by the Perls' reaction. (**F**) In a specimen in the rescue phase after 2 hours of anoxia accumulations of the residues of erythrocytes form white, roundish agglomerates that bulge from the surface of the spleen. Scale bar, 1 cm. (**G**) In the splenic tissue Perls' reaction stains the iron of haemolitic origin in blue. Scale bar 200 µm. The arrows indicate macrophages filled by fragments of erythrocytes (pinkish dots).

as long as the concentration of oxygen in the chamber did not fall well below 0.4 ppm, the oxygen consumption remained constant and the animals did not manifest any problems related to extracting the gas from the environment. In repeated trials using the method of Stewart *et al.* [40] and Ultsch *et al.* [41] of bubbling nitrogen through the water in an open container, we never succeeded in lowering its oxygen content to below 0.4 ppm; we therefore consider this method unsuitable for truly anoxic treatment.

Elementary principles of physics and physiology exclude the possibility that "residual oxygen" may persist in circulating blood of specimens under anoxia [42-44]. Thus, the substantial amounts of oxygen in the blood stream (even after 48 hours of total anoxia) do not only demonstrate the existence of the hepatic mechanism for producing this molecule but also its effectiveness. On the other hand, the guarantee that the oxygen found in the blood drawn from the arterial cone of the newts under anoxia was exclusively endogenous comes from the precautions taken before sampling. Paraffin oil acts as diffusion barrier to O_2 [28] and directly transferring the animals from anoxic water to paraffin rules out the possibility of contact with air, including any that the operator could bring in water with his manoeuvres. The total lack of oxygen noticed in the blood drawn from the left atrium of the group ANOXIA 48h B testifies the validity of our procedure.

We must observe that Stewart *et al.* [40] and Ultsch *et al.* [41] have not found oxygen in the blood collected "from the heart" of frogs submitted to anoxia (the exact point of withdrawal is never mentioned). There is a considerable phylogenetic distance between anurans and urodeles, but not to the extent that it can justify such a profound physiological difference in the two orders of amphibians considering that frogs tolerate long periods of anoxia as well and their livers also contain melanin. We believe that the differences in the results between frogs and newts depend on the point where the blood samples were drawn. The "arterial" blood collected from the left atrium and coming from the lungs in an anoxic environment cannot contain any trace of oxygen - like we have seen in our newts. However in anoxic specimens, we have found O_2 in the blood taken from the arterial cone, which conveys the whole cardiac output, both arterial and venous. The O_2 was in the latter.

The Oxygenogenesis Mechanism: Acquisitions and Hypotheses

The biochemical origin of the molecular oxygen released in the anoxic newts' blood is a very complex problem, but if we consider that the elements in play are only lack of oxygen, hepatic parenchyma, and products of haemolytic crisis, we can individuate several clews. Hypoxia is known to induce, especially in the liver, the production of free radicals, the so-called ROS, reactive oxygen species [45-50], but the hepatocytes possess enzymes which neutralise the ROS just by producing molecular oxygen [51, 52] - essentially superoxide dismutase (SOD) and catalase. The Fe^{2+} released in large amounts during the haemolytic process and destined to oxidise into Fe^{3+} outside the cells [53] should be linked with this process as an electron donor. In short, the model we propose to justify oxygenogenesis under anoxia differs only in the purpose, not in the function activated by the hepatocytes. Instead of cells performing the task of neutralising ROS by releasing oxygen as a by-product, they should be considered as cells that exploit ROS to actually produce oxygen.

As for the substratum for producing ROS we remember that the erythrocytes of lower vertebrates are nucleated (and thus contain DNA), and that the degradation of purines produces superoxide ($\cdot O_2^-$). The use of purines released owing to haemolysis as a substratum for oxygenogenesis would explain very well why the cells of lungfishes and amphibians possess the highest values of DNA content tabulated for the vertebrates [54] - the cells of our newt, for example, contain seven-eight times as much DNA as do those of man [55, 56].

As we hypothesised in the Introduction, however, it is just the release of endogenous oxygen in the liver that makes the synthesis of melanin possible in this seat; but the results of our experiments can also explain when and why this last process takes place.

In a previous study [18], we found that the hepatic melanin in newts is not "eumelanin", *i.e.* a pure polyindole [12], but a mixed polymer rich in nitrogen due to the incorporation of large amounts of purines coming from the nuclear DNA of erythrocytes disrupted during anoxic haemolysis. Thus, the hepatic melanogenesis can be recognised as having three essential functions to perform when anoxic animals return to a normoxic environment and have to recover their normal metabolic activities. In the rescue phase, with the oxidative synthesis of the pigment: a) blood hyperoxia resulting from the sum of endogenously produced oxygen and that assumed from the environment is cancelled out; b) the purines released owing to haemolysis are swiftly eliminated; c) ROS are neutralised, thanks to the "scavenger" effect of the polymerised pigment, thus avoiding the cytological damages they can cause when the oxygenogenesis reactions stop with the arrival of oxygen from the outside. Note that Sichel *et al.* [7] already proposed the scavenger function of melanin for superoxides on the basis of its chemical properties and the constant ratio between the amounts of pigment and SOD activity - see also Geremia *et al.* [57]. In key with this interpretation, anoxic animals exposed to the air after 16 hours have high amounts of hepatic melanin, but no longer have uric acid in their plasma or present the excess of oxygen that we have found after only 6 hours from their return to the air.

CONCLUSIONS

We can therefore conclude that our newts, and more generally speaking all lower vertebrates whose livers show the presence of melanin, possess a physiological mechanism that controls the production of molecular oxygen. The liver is a sort of "aqualung" activated by haemolysis and inactivated by melanogenesis - or however in close temporal relation with these two events.

Identification of this physiological mechanism, apart from providing a functional meaning to hepatic melanogenesis, allows a more correct interpretation of some well-known phenomena that in our opinion have not yet found satisfactory explanations. The first consideration is that (with the possible exception of very particular metabolic conditions as the hibernation [58, 59]) neurons capable of surviving anoxia by relying only on anaerobic glycolysis cannot exist, not even in lower vertebrates, as we have accepted until now

[36, 60]. An active animal will in any case tend to maintain a constant metabolic rate even under anoxia, integrating oxygenogenesis with anaerobic glycolysis which therefore is a complementary and not an alternative source of energy (see also Bennett and Licht [37]). A far more important consideration, from the biological-evolutionistic point of view, is that the incomplete separation between pulmonary and systemic circulation in amphibians is not the peculiar answer to a physiological problem or an incomplete evolutionary step (see Foxon [60], Farrel [61]); it is a functional requirement. A single ventricle allows the heart to distribute oxygenated blood to the brain and other tissues under all respiratory conditions. When breathing in air, the oxygenated blood reaches the ventricle mainly from the pulmonary veins through the left atrium [61, 62], but in normoxic water it comes exclusively from the skin and in anoxia exclusively from the liver taking the path that leads to the right atrium. The exchange between pulmonary and systemic blood flow cannot take place in a heart with two separate ventricles.

We also have to review the categories of "oxyregulators" and "oxyconformers" animals as defined by Boutilier [63]. It appears that lower vertebrates, until now defined as oxyconformers, *i.e.* "whose energy demands decrease with decreasing availability of O_2", have a physiological mechanism at their disposal that furnishes the molecule biochemically and allows maintaining fairly well sustained metabolic levels for long periods even under anoxia. Resorting to the energy savings identified by Hochachka *et al.* [22] - suspension of proteosynthesis, metabolic depression, channel arrest - is another resource to consider seriously, but is not the only way of postponing the catastrophic phases of the anoxic crises.

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SCOPE OF THE ARTICLE

The paper identifies a physiological mechanism that releases molecular oxygen in the bloodstream of the newt, and demonstrates the true role of the hepatic melanin. The authors believe that the discovery in vertebrates of physiological processes that has escaped all previous scientific analyses is an exceptional event, with deep cultural implications.

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