THE ACTIVITIES OF ANTIOXIDANT ENZYMES AND LIPID PEROXIDATION LEVELS IN RANA RIDIBUNDA RIDIBUNDA'S LIVER, HEART, LUNG, MUSCLE AND ERYTHROCYTES

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ABSTRACT

The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) and lipid peroxidation levels were investigated in the five vital tissues; liver, heart, lung, muscle, erythrocytes of predominantly aquatic amphibian Rana ridibunda ridibunda and the results obtained compared with the predominantly terrestrial amphibian Bufo viridis. These parameters related to antioxidant system and lipid peroxidation levels were also determined dependent on the metamorphosis period. SOD and CAT activity variations during the development period in B.viridis were higher than in R.r.ridibunda. CAT activity in R.r.ridibunda did not significantly change ($p>0,001$) until the 5th week and then increased, whereas in B.viridis it increased after the 3rd week. In contrast to the rise in the antioxidant enzyme activities, The minimum LPO levels in B.viridis and R.r.ridibunda were 23 ± 1.15 and 146 ± 7.3 nmol MDA/g tissue, at the 8th week, respectively. All antioxidant enzyme activities in tissues of adult B.viridis were higher than in R.r.ridibunda. When the season were concerned, all antioxidant enzymes were higher in activie season than in hibernation season. Accordingly, lipid peroxidation levels were also lower in hibernation period than levels in active period. When the body legnth were concerned, all antioxidant enzymes activities of 5-7 cm frogs of both species were higher than in 3-5 cm (young) specimens. While maximum SOD and CAT activities were determined in liver of B.viridis in the active period as 46 ±2.3 and 2631 ± 130.1 IU/mg, respectively, maximum GSH-Px activity was the highest in the heart of B.viridis 40.2 ± 1.5 IU/mg in the active period. When the membrane Lipid peroxidation levels were concerned, the tissues which having maximum antioxidant enzymes also had lower lipid peroxidation levels. According to results, in the war against to oxygen derived free radicals, B.viridis is more succesful than R.r.ridibunda since its higher antioxidant enzyme activities and lower lipid peroxidation levels.

ÖZET

Antioksidan enzimler superoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GSH-Px) ve lipid peroksidasyon düzeyleri sucul amfibi Rana ridibunda ridibunda'nın beş önemli dokusu;karaciğer, kalp, akciğer, kas ve eritrositlerinde incelendi, elde edilen sonuçlar karasal amfibi Bufo viridis dokularıylşa karşılaştırıldı. Ayrıca, antioksidan sistem ve lipid peroksidasyona bağlı olan parametreler yine bu kurbağa türlerinin metamorfoz sürecine bağımlı olarak ta incelendi. Gelişim peryodu boyunca B.viridis'teki SOD ve CAT aktiviteleri R.r.ridibunda larvalarındakilerden yüksekti. R.r.ridibunda larvalarının CAT aktivitesi 5.haftaya kadar anlamlı bir değişme göstermeyip ($p>0.001$) bu haftadan sonra artışa geçti. B.viridiste ise CAT enzim aktivitesi 3.hafta sonrası artış eğilimine geçti. Antioksidan enzim aktivitelerindeki artışın tersine, lipid peroksidasyon düzeyleri azalma eğilimi gösterdi (8.haftadaki LPO değerleri; R.r.ridibunda için 23 ± 1.15 ve B.viridis için 146 ± 7.3 nmol MDA/ g yaş ağ.) . B.viridis'in tüm dokularındaki antioksidan enzim aktiviteleri R.r.ridibundaya kıyasla daha yüksek bulundu. Sezon göz önüne alındığında, aktif sezondaki antioksidan enzim aktiviteleri kış uykusu periyodundan daha yüksekti. Buna bağlı olarak, kış uykusu periyodundaki LPO düzeyleri ise aktif sezondan daha düşük olarak saptandı. Yine vücut uzunluğu ele alındığında ise, 5-7 cm lik kurbağaların antioksidan enzim aktiviteleri 3-5 cm lik (genç) kurbağalarınkinden daha yüksek olarak bulundu. Maksimum SOD ve CAT aktivitesi aktif peryottaki B.viridis'in karaciğerinde 46 ± 2.3 ve 2631 ± 130.1 IU/mg olarak ölçülürken, en yüksek GSH-Px aktivitesi B.viridis'in kalbinde 40.2 IU/mg olarak ölçülmüştür. Yine LPO düzeylerine bakıldığında, maksimum antioksidan enzim aktivitesine sahip dokuların diğer dokulara kıyasla kısmen daha düşük LPO düzeylerine sahip oldukları gözlendi. Sonuçlara göre, oksijen kaynaklı serbest radikallere karşı yapılan metabolik savaşta, B.viridis sahip olduğu yüksek antioksidan enzim aktiviteleri ve düşük LPO düzeyleri yüzünden daha başarılı olduğu gözlenmiştir.

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INTRODUCTION

1.1. Description of the Species

Anura also called SALIENTIA one of the major extant orders of the class Amphibia. It includes the frogs and toads, which, because of their wide distribution, are known by most people around the world. The name frog is commonly applied to those forms with long legs and smooth, mucus-covered skins, toad being used for a variety of robust, short-legged anurans, especially those with rough skins. The name toad is applied so unevenly that one member of a family may be called a toad and a closely related member a frog. The familiar members of the family Bufonidae may be distinguished as "true toads." In this article "frog" is applied generally to members of the Anura and "toad" to those for which it has traditionally been used.

Frogs are used as teaching tools from grade school through college. One of the first biology lessons many children receive is through the rearing of the larvae, known as tadpoles or pollywogs, in science classes. Students become familiar with frog anatomy and embryology in biology courses. People in various parts of the world eat frog legs, and some kinds of toads are used in insect control. Certain South American Indians use the poisonous secretions of some kinds of frogs for poison arrows, and now biochemists are studying the possible medical uses of the constituents of the poison. The biologist interested in evolution finds a vast array of interesting, and often perplexing, problems in the study of frogs, a highly specialized group of amphibians.

1.1.1. General Features

1.1.1.1.Size Range and Diversity of Structure

Although all frogs are readily recognizable, there are great varieties of sizes and of structural modifications. Many frogs are tiny animals; perhaps the smallest is the Brazilian Psyllophryne didactyla, adults of which measure 9.8 millimetres (0.39 inch) in body length (with legs drawn in), whereas the West African goliath frog, Conraua goliath, has a body length of nearly 300 millimetres. Many anurans have smooth, moist skins. Toads of the genus Bufo are familiar as "warty" amphibians, the skin being highly glandular and covered with tubercles. Frogs of many other families have rough, tubercular skins, usually an adaptation for life in the less humid environments. The opposite extreme is found in the small arboreal frogs of the tropical American family Centrolenidae, in which the skin on the underside is thin and transparent, and the heart and viscera can be seen through the skin.

Most frogs move by leaping. The long and powerful hindlimbs are straightened rapidly from the crouching position, propelling the frog through the air. Many arboreal frogs, especially members of the family Hylidae, have adhesive disks on the ends of the fingers and toes and leap from branch to branch or from leaf to leaf. Members of the families Bufonidae, Rhinophrynidae, and Microhylidae and certain burrowing species in other families have relatively short hindlimbs and move forward by series of short hops. Some bufonids actually walk instead of hopping. Highly modified members of the hylid subfamily Phyllomedusinae have opposable digits on the hands and feet and walk slowly along branches, deliberately grasping the branch in the manner of tiny lemurs. Many kinds of frogs have membranous webbing between the fingers and toes; in the aquatic species the webbing on the feet aids in swimming. The extreme in this specialization is seen in the aquatic family Pipidae. Members of that family normally never leave the water. In regions of the Earth subjected to long dry periods, frogs must seek cover to avoid desiccation and have behavioral and

structural adaptations to conserve water.Although many frogs are unimpressively coloured, some species are brilliantly marked. The most common colours are brown, gray, green, and yellow. Uniformly coloured frogs are the exception rather than the rule. The markings of a frog may seem bold when observed out of the natural habitat, but they usually are concealing or disruptive when the frog is in its environment.

1.1.1.2. Distribution and Abundance

Because of their morphological and physiological adaptations, frogs are able to inhabit most regions of the world except the extremely cold landmasses at high latitudes and some oceanic islands that they have been unable to colonize because of the barriers provided by salt water. Frogs live in desert regions below sea level and in montane areas up to elevations above 4,560 metres (15,000 feet). Some members of the genus Rana live north of the Arctic Circle. Although widely distributed on the Earth, frogs are most diverse and abundant in the tropics, and 13 of the 24 families are restricted to the tropics. In most temperate areas of the world, the number of species of frogs at any one locality is usually fewer than 10, but in the tropics, especially in rain forests, the number of species is much greater. At one locality in the upper Amazon basin in eastern Ecuador, 83 species are known to occur, about the same number as is known for all of the United States.
In a complex environment such as a tropical rain forest, the large number of species of frogs partition the environmental resources in a variety of ways. In the humid tropics, frogs can be active throughout the year, but many species are seasonal in their breeding activity. Various kinds of sites and different seasons are used for calling and egg laying; such temporal and spatial separation avoids interspecific competition. Frogs feed mostly on insects and other invertebrates, and the abundance of food in tropical rain forests probably places no competitive restrictions on this aspect of environmental resources. Some large species eat vertebrates, including small rodents and other frogs

1.1.1.3. Reproduction and Breeding Behaviour

The breeding behaviour is one of the most distinctive attributes of the Anura. Because the eggs can develop only under moist conditions, most frogs place their eggs in bodies of fresh water. Many species congregate in large numbers at temporary pools for short breeding seasons. Others breed along the mountain streams where they live year-round. In the latter species and in those that breed on land, there is no great concentration of breeding individuals at one place. In all cases, the mating call produced by the male attracts females to the breeding site. It has been observed in the field and in the laboratory that the females can discriminate between mating calls of their own species and those of other species. At a communal breeding site, such as a pond, swamp, or stream, differences in specific calling sites of the males help the frogs to maintain their identities. Differences in mating calls, however, constitute the principal premating isolating mechanism that prevents hybridization of closely related species living in the same area and breeding at the same time and place. Frogs have rather simple vocal cords, in most species a pair of slits in the floor of the mouth opening into a vocal pouch. Air is forced from the lungs over the vocal cords, causing them to vibrate and thus produce sound of a given pitch and pulsation. The air passes into the vocal pouch, which, when inflated, acts as a resonating chamber emphasizing the same frequency or one of its harmonics. In this manner, different kinds of frogs produce different calls.

Females move toward and locate calling males. Once the male clasps the female in a copulatory embrace called amplexus, she selects the site for depositing the eggs. In the primitive frogs (superfamilies Discoglossoidea, Pipoidea, and Pelobatoidea) the male grasps the female from above and around the waist (inguinal amplexus), whereas in the advanced frogs (superfamilies Bufonoidea and Ranoidea) the position is shifted anteriorly to the armpits (axillary amplexus). The latter position brings the cloacae of the amplectic pair into closer proximity and presumably ensures more efficient fertilization.

Most frogs deposit their eggs in quiet water as clumps, surface films, strings, or as individual eggs. The eggs may be freely suspended in the water or attached to sticks or submerged vegetation. Some frogs lay their eggs in streams, characteristically firmly attached to the lee sides or undersides of rocks where they are not subject to the current. The large pond-breeding frogs of the genus Rana and toads of the genus Bufo apparently produce more eggs than any other anurans. More than 10,000 eggs have been estimated in one clutch of the North American bullfrog, Rana catesbeiana. The habit of spreading the eggs as a film on the surface of the water apparently is an adaptation for oviposition in shallow temporary pools and allows the eggs to develop in the most highly oxygenated part of the pool. This type of egg deposition is characteristic of several groups of tree frogs, family Hylidae, in the American tropics, one of which, Smilisca baudinii, is known to lay more than 3,000 eggs. Frogs breeding in cascading mountain streams lay far fewer eggs, usually no more than 200.

The problem of fertilization of eggs in rapidly flowing water has been overcome by various modifications. Some stream-breeding hylids have long cloacal tubes so that the semen can be directed onto the eggs as they emerge. Some other hylids have huge testes, which apparently produce vast quantities of sperm, helping to ensure fertilization. Males of the North American tailed frog, Ascaphus truei, have an extension of the cloaca that functions as a copulatory organ (the "tail") to introduce sperm into the female's cloaca.

Most frogs are considered to be placid animals, but recent observations have shown that some species exhibit aggressive tendencies, especially at breeding time. Male bullfrogs (Rana catesbeiana) and green frogs (Rana clamitans) defend calling territories against intrusion by other males by kicking, bumping, and biting. The South American nest-building hylid, Hyla faber, has a long, sharp spine on the thumb with which males wound each other when wrestling. The small Central American Dendrobates pumilio calls from leaves of herbaceous plants. Intrusion into a territory of one calling male by another results in a wrestling match that terminates only after one male has been thrown off the leaf.

Males of the Central American dendrobatid Colostethus inguinalis have calling sites on boulders in streams. The intrusion by another male results in the resident uttering a territorial call, and, if the intruder does not leave, the resident charges him, attempting to butt him off the boulder. Females of the Venezuelan Colostethus trinitatus wrestle in defense of territories in a streambed. Males of at least three South American species of Hyla build basinlike nests, 25 to 30 centimetres (10 to 12 inches) wide and 2 to 5 centimetres deep, in the mud of riverbanks. Water seeps into the basin, providing a medium for the eggs and young. Calling, mating, and oviposition take place in the nest, and the tadpoles undergo their development in the nests. Some frogs in the families Leptodactylidae, Ranidae, and Microhylidae build froth nests. The small, toadlike leptodactylids of the genus Physalaemus breed in small, shallow pools. Amplexus is axillary, and the pair floats on the water; as the female exudes the eggs, the male emits semen and kicks vigorously with his hind legs. The result is a frothy mixture of water, air, eggs, and semen, which floats on the water. This meringuelike nest is about 7.5 to 10.0 centimetres in diameter and about 5 centimetres deep. The outer surfaces exposed to the air harden and form a crust covering the moist interior in which the eggs are randomly distributed. Upon hatching, the tadpoles wriggle down through the decaying froth into the water.

1.1.1.4. Metamorphosis

Most frogs have an aquatic, free-swimming larval stage (tadpole). After a period of growth, the tadpole undergoes metamorphosis, in which the tail is lost and limbs appear. These are only two of the most obvious changes that take place. Tadpoles have a cartilaginous skeleton, thin nonglandular skin, and a long, coiled intestine; they lack jaws, lungs, and eyelids. Among the first changes that take place is the appearance of hindlimb buds, which grow and develop into differentiated hindlimbs, complete with toes, webbing, and tubercles. Much later, the front limbs emerge through the skin; in contrast to the hindlimbs, the forelimbs develop before they emerge. At the time of emergence of the forelimbs, the tail begins to shrink, being absorbed by the body. The tadpole mouth begins to

change; as the horny denticles and papillae, if present, disappear, the jaws and true teeth develop. The eyelids develop, and mucous glands form in the skin. The vertebral column and limb bones ossify, and the adult digestive system differentiates as the long, coiled intestine shrinks to the short, thick-walled, folded intestine of the adult. These radical changes are equaled in the animal kingdom only by the metamorphosis found in insects.

Just how and where the changes from larva to adult take place is highly varied and is one of the most fascinating aspects of the study of frogs. The differences in modes of life history reflect varied environmental conditions. In various evolutionary lines in frogs, there is a strong tendency toward an escape from the ties that bind the less advanced frogs to the water for breeding.

The tadpoles of the pond breeders characteristically have rather large bodies and deep caudal (tail) fins, which in some have a terminal extension, as do the familiar swordtail fishes. The mouths are relatively small, either at the end of the snout or on the underside, and usually contain rather weak denticles. These tadpoles swim easily in the quiet water and feed on attached and free-floating vegetation, including algae. In contrast, the stream tadpoles have depressed bodies, long muscular tails, and shallow caudal fins. The mouth is relatively large and usually contains many rows of strong denticles. In highly modified stream tadpoles, the mouth is ventral and modified as an oral sucker, with which the tadpole anchors itself to stones in the stream. Such tadpoles move slowly across stones, gnawing off moss or algae as they move. It is conceivable that a tadpole can complete its larval life on a single large stone.

Most tadpoles complete their development in two or three months, but there are notable exceptions. Tadpoles of spadefoot toads, genus Scaphiopus, develop in temporary rain pools in arid parts of North America, where it is imperative for the tadpoles to complete their development before the pools dry up. Some Scaphiopus tadpoles metamorphose about two weeks after hatching. In the northern part of its range in North America, the tadpoles of the bullfrog Rana catesbeiana require three years to undergo their development.

Some tree frogs of the family Hylidae deposit their eggs in water that has pooled in parts of trees. Several tropical species of Hyla lay their eggs in the water held in the overlapping bases of leaves of epiphytic bromeliads high in trees. Their tadpoles, which are slender with long, muscular tails, develop in small quantities of water high above the ground. The Mexican hylid, Anotheca spinosa, lays its eggs in bromeliads or water-filled cavities in trees. The small tadpoles, like those of Hyla, feed on aquatic insect larvae, such as mosquitoes, but the larger tadpoles of Anotheca apparently feed only on the eggs of frogs.

A modification of the basic pattern of depositing aquatic eggs is the placement of eggs on vegetation above water; this pattern occurs in some arboreal hylids and ranids and in all species in the family Centrolenidae. Hyla ebraccata, a small Central American tree frog, deposits its eggs in a single layer on the upper surfaces of horizontal leaves just a few inches above the pond. Upon hatching, the tadpoles wriggle to the edge of the leaf and drop into the water. The Mexican Hyla thorectes suspends 10 to 14 eggs on ferns overhanging cascading mountain streams. The phyllomedusine hylids in the American tropics suspend clutches of eggs from leaves or stems above ponds. Males call from trees; once a female has been attracted and amplexus takes place, the male placidly hangs onto the back of the female as she descends to the pond and absorbs water. This accomplished, she climbs into a tree, selects an oviposition site, and deposits eggs until her water supply is depleted. She again descends to the pond and repeats the performance at a different site until the entire complement of eggs is deposited. Upon hatching, the tadpoles drop into the pond below. Most of the tree frogs of the family Centrolenidae are less than 2.5 centimetres long. Males call from leaves of trees or bushes over cascading mountain streams in the American tropics. Individuals return to the same leaf night after night. Attracted females are clasped on the leaf, and egg deposition takes place there immediately. A highly successful male may have three or four egg clutches on his leaf, each consisting of only about two dozen eggs. Upon hatching, the tadpoles drop into the streambed; if a tadpole lands on a stone, it flips about vigorously until it falls into the water, where it hides in the loose gravel on the bottom of the stream.

1.1.2. Alternative Reproductive Strategies

Many kinds of frogs lay their eggs on land and subsequently transport the tadpoles to water. The ranid genus Sooglossus of the Seychelles and all members of the family Dendrobatidae in the American tropics have terrestrial eggs. Upon hatching, the tadpoles adhere to the backs of adults, usually males. The exact means of attachment is not known. The frogs carry the tadpoles to streams, bromeliads, or pools of water in logs or stumps where the tadpoles complete their development. The most unusual example of tadpole care known is that exhibited by the mouth-brooding frog, or Darwin's toad, Rhinoderma darwinii, in southern South America. An amplectic pair deposits 20 to 30 eggs on moist ground. Several males gather around the eggs, and, when the eggs are about ready to hatch, with the embryos moving, each male picks up some eggs with his tongue. The eggs pass through the vocal slits in the floor of the mouth and into the vocal sac. The eggs hatch, and the larvae complete their development in the large vocal sac. Upon metamorphosis, the young frogs emerge from the male's mouth.

The European midwife toad, Alytes obstetricans, displays a peculiar breeding habit. Inguinal amplexus takes place on land; at the time of oviposition, the female extends her legs to form a receptacle for the string of 20 to 60 eggs. After fertilizing the eggs, the male moves forward on the back of the female and pushes his legs into the string of eggs until they are wound around his waist and legs. Then the female departs. The male carries the eggs with him on land until they are ready to hatch, at which time he moves to a pond where the eggs hatch and complete their development.

The hylid Gastrotheca marsupiata, one of several so-called marsupial frogs, lives in the high Andes of South America. In amplexus the male exudes a quantity of semen, which flows into the pouch. The female extrudes eggs a few at a time; these are pushed into the pouch by the male, who uses the hindfeet to catch and push the eggs. The eggs are fertilized in the pouch, where they hatch and the tadpoles begin their development. Subsequently, the female moves to a pond,

where the tadpoles emerge from the pouch and complete their development in the water.

In each of the above instances of parental care, there is a trend away from the aquatic environment. Far fewer eggs (fewer than 50) are laid in comparison with those species depositing eggs in the water. The bonds with the aquatic environment have been partially broken, for, although the tadpoles must develop there, the eggs are effectively terrestrial; however, they are not truly so, because they lack the necessary embryonic membranes (allantois and amnion) to maintain physiological balance, and they also have no shell. Consequently, if they are to survive and develop, the eggs must be maintained in moist places, such as damp soil or a part of the parental body. Water and waste products are transported through the membranes by osmosis.

The next evolutionary step in mode of life history is the elimination of the larval stage, thereby completely severing the ties with the aquatic environment. Direct development of the egg, in which the larvae undergo their development within the egg membranes and emerge as tiny froglets, occurs in many species, scattered in 12 families (Leiopelmatidae, Pipidae, Leptodactylidae, Bufonidae, Brachycephalidae, Hylidae, Myobatrachidae, Sooglossidae, Arthroleptidae, Mantellidae, Ranidae, and Microhylidae). Typical direct development of terrestrial eggs occurs in the many species of the leptodactylid genus Eleutherodactylus of Central and South America and the West Indies. During axillary amplexus, the female deposits a clutch of eggs in a moist place (beneath a log or stone, amid leaf litter, in a rotting stump, in moss, or in a bromeliad). The parents depart, leaving the eggs to develop and subsequently hatch. In some Eleutherodactylus species and in the New Zealand leiopelmatid Leiopelma hochstetteri, the hatching froglet still has a tail. In Leiopelma, at least, vigorous thrusts of the tail are used to rupture the egg membranes. Soon after hatching, the tail is completely absorbed.

Brooding of terrestrial eggs is known in a few species. Females of two species of Eleutherodactylus that lay their eggs on leaves of bushes or trees sit on the eggs. Apparently this brooding serves to prevent desiccation of the eggs by dry winds. Females of the Papuan microhylid Sphenophryne lay their few eggs beneath stones or logs and sit on top of them until they hatch.

Direct development occurs in several species of hylid marsupial frogs (Gastrotheca) living in mountain rain forests in northwestern South America. In these frogs, amplexus is axillary, and the female raises her cloaca so that the eggs, which are extruded one at a time, roll forward on her back and into the pouch. There the eggs develop into frogs. Large, external, gill-like structures envelop the developing embryo. These structures, which are attached to the throat of the embryo by a pair of cords, apparently function in respiration. These frogs live high in trees and complete their life cycle without descending to the ground. Thus they are rare in collections, and their biology is poorly known.

Some other South American genera of Hylidae also exhibit the phenomenon of direct development of eggs carried on the backs of the females. In Flectonotus and Fritziana the eggs are contained in one large basinlike depression in the back, whereas in others each egg occupies its own individual depression. In Hemiphractus gill-like structures and cords similar to those in Gastrotheca are present. At hatching, the expanded gill adheres to the modified skin of the maternal depression and is attached to the young by the pair of cords. The female carries the young until they are sufficiently well developed to care for themselves. The manner of detachment of gill from female and young is unknown. The strictly aquatic Surinam toad Pipa pipa of northern South America also has direct development, in this case in the water. Eggs are carried in individual depressions in the back of the female. Amplexus is inguinal, and the pair rests on the bottom of the pond. The female initiates vertical circular turnovers, at the height of which she extrudes a few eggs. These are fertilized, fall against the belly of the then upside-down male, and are pushed forward onto the female's back, where they adhere and become enclosed in tissue. When developed, the young frogs emerge from the skin of the female's back.

The most advanced form of reproduction known in frogs takes place in the small African bufonids of the genus Nectophrynoides. By some unknown means, fertilization is internal, and the young are born alive. It is noteworthy that the evolution of live birth has taken place independently in all three living orders of amphibians, for this phenomenon also occurs in salamanders and caecilians.

1.1.3.Feeding Habits

The great majority of frogs feed on insects, other small arthropods, or worms, but some larger species eat vertebrates. The South American leptodactylid Ceratophrys varius and the large bufonid Bufo marinus eat other frogs and small rodents. The superficially similar Solomon Island ranid, Ceratobatrachus guentheri, and the South American hylids, Hemiphractus, eat other frogs. Large North American bullfrogs, Rana catesbeiana, have been reported to consume other frogs, mice, small snakes, and even small turtles.

1.1.4. Critical Appraisal

Modern authorities do not agree on all aspects of anuran classification, and further study is needed to clarify the relationships of certain groups. The most widely used classification above the family level is that established by G.K. Noble in 1931, in which five suborders were recognized, based on vertebral characteristics. The superfamilies given above are not exactly equivalent to the suborders of Noble, the following modifications being most noteworthy: (1) The names Ascaphidae and Leiopelmatidae have been used interchangeably and recognized as distinct by various authorities. (2) The family Myobatrachidae includes what were formerly recognized as Old World members of the Leptodactylidae, a family now considered restricted to the New World. (3) The Brachycephalidae, as recognized by Noble, included the dendrobatids and the peculiar mouthbrooding frog (Rhinoderma), now considered distinct families, as well as several genera (e.g., Atelopus) currently placed in the Bufonidae. Most authorities now restrict the family Brachycephalidae to two genera, Brachycephalus and Psyllophryne. (4) The family Atelopodidae (sometimes

spelled Atelopidae) of many authors is now relegated to the Bufonidae. (5) Rhinoderma, which other authors have placed in the Atelopodidae, Leptodactylidae, and Bufonidae, is now given family status. (6) The relationships of the Microhylidae are uncertain; this family may have had an evolutionary history entirely independent from the ranoids, with which they have been associated by some authorities.

Chapter Two

OXIDANTS AND ANTIOXIDANTS

2.1. Oxidants and Antioxidants in Living Organism

Oxygen is vital for life, yet it is also potentially toxic. To all aerobic organisms it is vital for the production of energy by its controlled reduction to water, yet to most forms of life it caused numerous manifestations of toxicity at greater than normal partial pressure. And also, there is a very strong thermodynamic driving force for the reactions between oxygen and biochemical compounds in the body, like proteins, carbohydrates, lipids and nucleic acids. If such reactions go to completion, water, carbon dioxide and a number of waste products are formed as end products concomitantly with the release of large amounts of energy. Fortunately such reactions occur spontaneously very slowly due to reaction barriers (Hallivel, and Gutteridge, 1984). Oxidation of biological compounds is, however, the source of energy of living organisms. How ever, the direct four-step reduction of oxygen to water is almost unique, and when oxygen reacts spontaneously or catalysed by enzymes it is for mechanistic reasons forced to react one step at a time (Hallivel, and Gutteridge, 1984).

Free radicals are molecules or fragments of molecules containing unpaired electrons in their outer orbitals, which represent a free valence. Unpaired electrons tend to acquire a pair, therefore, free radicals are chemically highly reactive and have a short half-life. Free radical reactions are nonenzymatic chain reactions. Free radicals of oxygen play an important role in biology and medicine. They are generated from oxygen by excitation (singlet oxygen $'0_2$)or reduction (superoxide anion radical 02, hydrogen peroxide H_20_2, hydroxyl radical OH). Not all reactive products of the reduction or excitation of oxygen are free radicals (e.g. singlet oxygen, hydrogen peroxide), but their reactivity and reactions resemble those of

free radicals. These products, together with the true free radicals are called reactive oxygen intermediates (Feher, Csomos and Vereckei, 1987).

The importance of oxygen-derived radicals, reactive oxygen species, and antioxidants in health and disease is now recognized by every branch of medicine and biological science. Overwhelming evidence indicates that free radicals play a role in most major health problems of the industrialized world, including cardiovascular diseases, cancer, neurologic disease, and aging and that antioxidants play a critical role in weliness, health maintenance, and the prevention of chronic and degenerative disease. Oxidants also play a role in some aspects of health, as in the oxidative burst of neutrophyls and macrophage which allows them kill foreign organisms (Hallivel, and Gutteridge, 1984)

Potentially damaging oxygen free radicals can be generated within the body, by a variety of processes. The superoxide free radical, particular, is thought to be formed enzymically during oxygen reperfiision following ischemia, during phagocytosis or through the action of toxic chemicals (Asada et al, 1980).

It is clear that free radical reactions are amongst the panoply of mechanisms leading human cancer and this presents opportunities for intervention to prevent the processes of initiation, or progression. Certainly there is a growing body of preventive role in at least certain types of cancer (Bors et al, 1974).

Broadly speaking, there are three potential roles for free radicals and active states of oxygen in environmental carcinonesis: the agent itself may be a free radical or active state of oxygen; these active species may serve as the intermediary by which an environmental chemical produces genetic damage; or the metabolism of the chemical through a free radical dependent step may lead to the formation of a carcinogen (Farber, 1981).

2.2. The Production of Superoxide Radicals in Biological Systems

An EPR signal, characteristic For superoxide radical, was observed by the rapid-freezing technique in the oxidation at pH 10 of xanthine by dioxygen catalysed by xanthine oxidase (EC 1.2.23.2) (Biaglow et al, 1989). The enzymatic reduction of dioxygen by aldehyde oxidase (EC 1.2.3.1) produces also the superoxide radical.

The plasma-membrane bound superoxide-generating flavoprotein (superoxide synhetase) of human polymorphonuclear neutrophyls (PMN) has been solubilized by Triton X-100 (Beutler, 1989) and deoxycholate (Barkley, 1990) and purified. The enzyme catalyses the reaction:

$$2O_2 + NAD(P)H + H_2O \longrightarrow NAD(P) + 2O_2^{\cdot} + H_3O^+$$

NAD(P)H seems to react on the cytosolic side of the membrane, while the superoxide anion in generated at the outer side (Burdon, 1995).

Following a stimulation by the yeast cell wall extract zymosan, phagocytosing human leukocytes show an enhanced oxygen consumption, the cyanide-insensitive "respiratory burst", after a lag period of 30-40s. The production of superoxide anions, mesured by the reduction of Fe(III)-cytochrome c, followed the same course (Chance, 1979). The formation of superoxide radicals was directly demonstrated by spin trapping on stimulated human neutrophils (Chien, et al. 1979).

A similar enzymatic activity was found in stimulated pulmonar alveolar macrophages from humans (Cohen, et al. 1984), guinea pigs (Holian, Daniele, 1979), and rats (Hirai, Ueno, Ogawa, 1980), and in retinal pigment epithelial cells from chick embryos (Ueno, et al. 1979)

A cyanide-sensitive NAD(P)H dependent superoxide generating system was

described, however, for the nuclear membrane of hepatoma 22a ascites cells grown in mice (Crane at al,1980). The formation of superoxide has, morever, been reported for cytochrome P-450, diamine oxidase, flavoproteins, and peroxides (Fee, 1980).

Superoxide radicals can also be produced in the reduction of dioxygen by strong reducing agents, like iron-sulfur proteins, flavins, phenylhydrazine, semiquinones, and thiols (Fee, 1980; Bors, et al., 1974; Fridovich, 1974). Some of the reactions of dioxygen, like the herbicide paraquat (1,1 '-dimethyl-4,4'-bipyridinium dichloride) in Escherichia coli (Hassan, Fridovich, 1979) and with mouse lung microsomes (Cross et al,1987).

The production of superoxide radicals was also observed in the "autoxidation" of oxyhemoglobin the methemoglobin: with shark hemoglobin by induction of the oxidatin of adrenaline to adrenochrome (Gotoh, Shikama, 1976). Similarly with human hemoglobin a proton assisted displacement of superoxide by nucleophilic anions has been described (Wallace, Maxwell, Caughey, 1974), although the authors have considerably modified their first interpretation (Wallace, Caughey, 1979)

Finally superoxide radicals can also be generated photochemically in chioroplasts in the presence of ascorbate or of paraquat. The formation was demonstrated by spin trapping on illumination of spinach chloroplasts in the presence of oxygen and paraquat (Harbour, Bolton, 1975). Superoxide radicals are formed, morever, in the near-ultraviolet photooxidation of tryptophan, as indicated by the increase of the H_20_2 in the presence of SOD (Mccormick, Thomason, 1979,), and on irraditon in aerated solutions of protoporphyrin at 400 nm (Dormandy, 1978) and of melanin with light of320-600 nm (Felix, et al., 1978), as shown by spin trapping.

In aprotic solvents the superoxide anion is a very weak oxidant: the uptake of a proton from the substrate is followed by the dismutation of $H0_2$ (Sawyer, Gibian, 1979). Thus stable solutions of 0? can be prepared by controlled-potential coulometry (Fernandez-Sousa, 1976).

2.3. Control of Free Radical Reactions in Biological Systems

Since living organisms contain several free radicals under physiological conditions, there must exist innate mechanisms keeping their reactions under control in situations like electron transport, melanin production or photosynthesis. Molecular structures of vital importance like the cell membrane. DNA and proteins have constituents which are very sensitive to radical reaction, such as the a-methylene carbon atom ofPUFAs as well as allylic hydrogen, and the thymidine base (Feijoo et al, 1997).

Free radicals formed during electron transport are kept under control by the enzymes and carriers of the transport chain, which are bound to the membrane and are closely packed together. The PUFAs of the membrane are localized in its hydrophobic region, far from the site of electron transport, which takes place in the hydrophilic region. Thus the protection of PUFAs is inherent in membrane structure. This defence may be damaged if the cell membrane or mitochondria are injured, giving rise to pathological free radical reactions. PUFAs of the membrane phospholipids are "packaged" by cholesterol. Hydrogen bonds in membranes also provide protection against free radicals. However, hydrophobic chemical pathogens (e.g. CCl_4, DDT, halothane and some carcinogenic substances) can penetrate the hydrophobic region and initiate free radical reactions (Lohr et al,1995).

Some normally occurring free radicals are under control because they are intermediates formed during reactions of enzyme-substrate complexes. Owing to their fixed localization and short half-life, they have little chance to interact with other substances (Lopez Torres et al, 1988).

The rate of radical reactions is influenced by the amount of membrane unsaturated fatty acids and the degree of their unsaturation. These, in turn, depend to a lesser extent on the fat content of the diet, and predominantly on genetic factors (Demopoulos, 1973)

Catalase dismutases hydrogen peroxide. The enzyme is found in variable amounts in tissues in the body (Markiund, Westman, Lundaren, Roos, 1982), in general in peroxisomes together with hydrogen peroxide-producing oxidases. Frythrocytes contain much catalase. Glutathione peroxidases are intracellular enzymes catalyzing the reaction between glutathione and hydrogen peroxide or organic hydroperoxides. Two isoenzymes contain selenium (FlohI, 1982; Ursini, Mairino Gregolin, 1985) and this is the only known function of selenium in the body.

In the cytochrome oxidase complex, oxygen is directly reduced in four steps:

$$0_2 \quad +4e+ 4H \longrightarrow 2H_20$$

Nonenzymatic reduction occurs one step at a time, "Univalent Pathway":

$$0_2 \xrightarrow{e} \underset{\text{Superoxide anion radical}}{O_2} \xrightarrow{e} \underset{\text{hydrogen peroxide}}{H_20_2} \xrightarrow{e} \underset{\text{hydroxyl radical}}{OH^-} \xrightarrow{e} 2\,H_20$$

The Fenton reaction

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{+3} + OH + OH$$

The Haber-Weiss reaction:

$$H_20_2 + O_2 \xrightarrow[Fe]{Cu} OH + OH + 0_2$$

Reaction catalyzed by catalase:

$$H_20_2 + H_20_2 \longrightarrow O_2 + 2H_2O$$

Reaction catalyzed by glutathione peroxidase

$$\underset{\text{(or organic peroxide)}}{H_2O_2} + \underset{\text{reduced glutathione}}{2GSH} \longrightarrow \underset{\text{oxidized glutathione}}{GSSG} + 2H_20$$

A new enzyme reported in 1969 whose function is the disproportionation of superoxide radicals to oxygen and hydrogen peroxide and was named superoxide dismutase (SOD) QC 1.15.1.1) (Mccord and Fridovich, 1969,a):

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

SOD was subsequently found to be a universal enzyme which exists in three different metalloforms where each form incorporates a transition metal ion at the active site. SOD is not known to react catalytically with any substrate other than the superoxide radical. Prior to discovery of SOD many biochemists regarded the possible existence of oxygen radicals in the cellular environment with extreme scepticism. The proof of the existence of an enzyme which catalysed the removal of such a radical forced a change of view, and subsequently had profound and far reaching effects on the
study of reactive oxygen species in living biological systems (Asada, Kanematsu, Okaka, and Hayakawa, 1980; Gregory, Pennington, and Denz, 1990).

2.4. The Structure of Superoxide Dismutase

2.4.1.Isoenzymes of Superoxide Dismutase

Although the nature of the enzymatic activity of CUZnSOD was discovered over two decades ago, the protein had been known much earlier. It was first isolated as a copper protein from bovine erythrocyte (erythrocuprein) and liver (hepatocuprein) in 1938 (Mann, and Keilin, 1938). This was followed by the discovery of a whole family of copper-containing proteins that were present in various organs (Carrico, and Deutsch, 1969). Much later, as a the result of various

observations carried out by McCord and Fridovich (Mccord, and Fridovich, 1969) on the reduction of cytochrome C by xanthine oxidase reaction, erytrocuprein was identified as copper zinc superoxide dismutase. CuZnSOD is a soluble enzyme found mainly in the cytosol of eukaryotic cells. It has a molecular mass of 32000 and it consists one Cu^2 and one $Zn+^2$ in the active site. CuZnS0Ds are generally very stable enzymes, tolerating exposure to organic solvents and retaining activity in 8.0 M urea (Forman, and Fridovich, 1973) or in 2% SDS (Malinowski and Fridovich, 1979). CuZnSODs from some sources are not as stable in the presence of denaturants (Barra, et al. 1979; Nakano, Sato, and Takeuchi, 1995). In general, the properties and structure of CUZnSOD have been remarkably resistant to evolutionary modifications, and enzymes obtained from plants, fungi, birds and mammals are very similar (Halliwell, and Guneridge, 1989, Asada, Kanematsu, Okaka, and Hayakawa, 1980; Clarke, and Cowden, 1985; Docampo, 1990; Gregory, Pennington, and Denz, 1990; Fridovich, 1995; Fridovich, 1975).

CuZnSOD has also been isolated from several bacterial species, including Escherichia coli (Fridovich, 1975; Steinman, 1987; Steinman, and Ely, 1990; flenov, and Fridovich, 1994), and its presence has been indicated in some protozoan parasites (Michalski, and Prowse, 1991). Bacterial Cu,ZnSODs are most likely periplasmic (Stabel, Sha and Mayfield, 1994; Beck, Tabatat, and Mayfield, 1990) and have properties similar to those of eukaryotic CuZnSOD (Kroll, Langford, and Loynds, 1991). In E.coli, CuZnSOD seems to be important for aerobic growth in mutants that cannot produce MnSOD and FeSOD (Benov and Fridovich, 1994). It remains to be elucidated whether there are sources of 02 within the periplasm or whether the periplasmic SOD protects against extracellular source of 02 (Fridovich, 1995).

Manganese containing SOD was first isolated form E.coli, McCord, Fridovich, 1969) and has since been isolated from a wide range of bacteria (Steinman, 1982; Beyer, Imlay and Fridovich, 1991). The bacterial enzyme contains manganese in its active site (one Mn per subunit, in the resting state), usuafly has a molecular mass of 40000 to 46000, and is a dimer made up of identical subunits. High

molecular mass tetrameric MnSODs have been identified in several species of bacteria with molecular masses of 110000 to 140000 (Gregory and Dapper, 1980; Barkley and Gregory, 1990). MnSOD has also been obtained from mitochondria. The mitochondrial enzyme is strikingly similar to the MnSOD from prokaryotes, with the notable difference that it is always tetrameric (Asada, Kanematsu, Okaka, and Hayakawa, 1980; Steinman, 1982; Beyer, Imlay and Fridovich, 1991). MnSODs are much more susceptible to denaturatin by heat or chemicals (including solvents and detergents) than are CuZnSODs (Bannister, and Bannister, 1984).

Fe-containing SOD was first isolated from E. Coli and subsequently from many other bacteria (Halliwell, and Gun eridge, 1989; Fridovich, 1975). No animal tissues have been found to contain FeSOD of43 plant families examined, FeSOD was found in only three (Bridges and Salin, 1981). All known FeSODs are vey similar to MnSOD, and with few exceptions are dimeric with a subunit molecular mass of 23000 and with one atom of metal per subunit (Halliwell, and Gutteridge, 1989; Asada, Kanematsu, Okaka, and Hayakawa, 1980; Gregory, Pennington, and Denz, 1990; Fridovich, 1975). Like mitochondrial MnSOD, some bacterial FeSODs were found to be tetrameric (Kusunose, Ichihara, Noda and Kusunose, 1976). Some bacteria contain both MnSOD and FeSOD, whereas others contain only one the type of enzyme. In some bacterial strains, the type of SOD expressed appears to depend on the availability of a metal in the growth medium (Halliwell, and Gutteridge, 1989; Fridovich, 1986). MnSOD and FeSOD have very similar amino acid sequences and share a common polypeptide fold, which is completely that MnSOD and FeSOD are structural analogues. Yet these enzymes from E.coli exhibit substantial differences in their solution properties and absolute metal cofactor specificity (Carlioz, 1988).

2.4.2. The Inhibitors of SOD

Extracts of tissues or cells will ordinarily contain more than one type of SOD, and means for individually assaying SODs in such mixtures have been devised. These usually depend upon reagents that inhibit or inactivate one of the SODs,

while having no effect on the others. Thus cyanide inhibits CuZnSOD, but not FeSOD or MnSOD (Rotiho, Bray and Felden, 1972; Kanematsu, and Asada, 1978; Beauchamp and Fridovich, 1973). One may' use 5 mM CN to specifically suppress the activity of CuZnSOD in extracts (Ysebaert-Vanneste, and Vanneste, 1980). Hydrogen peroxide on the other hand, inactivates CuZnSOD (Fielden, F.M., Roberts, Bray and Rotiho, 1973; Beauchamp and Fridovich, 1973; Symonyan, and Nalbandyan, 1972) and FeSOD (Asada, et al, 1975), but has no effect on MnSOD (Asada, et al. 1975; Hodgson, and Fridovich, 1975). Alternately, MnSOD activity can be entirely eliminated by 2% sodium dodecyl sulfate, which has no effect on CuznSOD (Geller and Winge, 1983). And also, all of SODs are inhibited by sodium azide (Asada, et al. 1975)

The two active sites on the CuZnSOD enzyme function independently although separated subunits have not been found to be active. It is thought that the reaction of one copper-site with the substrate prevents the site on the other subunit from catalytic function, i.e. antico-operative interaction between the two sites (Rotiho et al., 1977). Cyanide completely inhibits CuZnSOD by binding to the inner co-ordination sphere of the copper (Fee and Gaber, 1972)

SOD is powerful enough to increase the rate of dismutation of 02 by several orders of magnitude at physiological pHs. The catalytic activity of Cu,ZnSOD has been shown by direct assay to be constant over the pH range 5-9.5 (Rotiho et al,, 1972; KIng et al., 1972) and to decrease rapidly above pH 10 where as the activity of MnSOD is substantially suppressed at the higher pH.

2.5. The Structure of Catalase

Catalases are metallo-enzymes that degrade H_2O_2 via the following reaction.

$$2\,H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

The decomposition of two molecules of H_2O_2 takes place in two steps. The first involves contact between the ferric state of the enzyme (Fe-E) and H_2O_2 to produce an intermediate from of the enzyme termed Compound I. Compound I than interacts with a second molecule of H_2O_2. The following summary interpretation has been presented (where of -E represent compound I) (Fita and Rossman 1985).

The H_2O_2 degrading ability of catalase has been known since early in this century. For decades there was persistent doubt among some scientist that this catalytic activity represented the in vivo function of the protein. This interesting parallel between the histories of catalase as a scavenger of a toxic oxygen by Product. H_2O_2.

As is the case with O_2, H_2O_2 is a ubiquitous product of aerobic metabolism. It is generated directly by numerous enzymatic and non-enzymatic cellular reaction (Farr and Kogoma 1991). It is also degradation product of O_2, H_2O_2 is implicated in damage to a diversity of biological molecules, including DNA. There is evidence from studies with E.coli that DNA is in fact one of the primary targets for H_2O_2mediated damage (Imlay and Linn 1987;Imlay and Linn 1988).This is supported by the conclusion that in E .coli the DNA repair enzyme RecA may be more important than catalase in protecting against H_2O_2-mediated damage cell death(Carlsson and Carpenter 1980).The precise mechanisms of H_2O_2 -mediated damage have remained illusive ,although it is accepted that one important pathway to damage involves interaction with O_2^- to produce hydroxyl radicals as described earlier(Farr and Kogoma 1991) .

Catalases from diverse organisms comprise a group of related proteins . Most catalases studied to date are homotetramers ,although hexameric forms have been reported from prokaryotes (Loewen and Switala 1986,1987).Substantial variation exists in terms of apoprotein size ranging from 55kDa in N.crassa and A.niger(Fowler et al.1993).Such variation is due to sequence differences in the

rather conserved core of the protein . Portions of the general catalase sequence exhibit high degrees of amino-acid conservation whereas other regions show very little conservation when for example fungal sequences are compared with those from plants or animals(von Ossowski et al.1993).Nearly all catalases studied to date possess a heme prosthetic group, wherein iron is central to catalysis. Catalases with manganese, instead of iron, as the reactive metal have been reported from certain prokaryotes. To our knowledge, they have not yet been studied at either the amino-acid or nucleotide sequence level. They possess subunit sizes (28-34kDa) substantially smaller than those of heme catalases however, and therefore may not be members of the heme catalase family.

2.5.1.Inhibitors of CAT

Inhibition by ascorbate alone as well as with Cu^{2+} has been shown by Orr (1967a and b). Freezing and lyophilization cause inactivation (Tanford and Lovrien 1962; Deisseroth and Dounce 1967). Mitchell and Anderson (1965) indicate catalases to be inactivated by sunlight under aerobic conditions. Catalase inactivation by peroxide has been reported on by Altomare et al. (1974).

Stability: All preparations are stable for 6-12 months when stored at 5deg.C. Do not freeze liquid preparations.

2.6. Glutathione Peroxidase

Glutathione (γ-glutamylcysteinylglycine: GSH), discovered in 1921 by Hopkins , is now widely recognized for its important role in protecting cells from free radicals, peroxide, and xenobiotic-mediated injury. This important thiol is synthesized by the consecutive action of two enzymes γ-glutamylcysteine and glutathione synthetases (Hidalgo et al,1990). It has been known that GSH is present in almost all organisms and serves as a reductant in numerous biochemical reactions, including counteraction of oxidative events and protection of the thiol groups of intracellular proteins (Harman et al, 1986)

The active oxygen species which are produced in the cells contribute to the pathogenesis of several diseases (Lawrence et al, 1976). Both prokaryotes and eukaryotes have defensive mechanisms against the toxicity shown by active oxygen species (Szatrowski, 1991). One important cellular antioxidant enzyme is glutathione peroxidase (GSHPx). first reported in 1957 by Mills. which catalyzes the reduction of hydroperoxides to their metabolizable hydroxyl forms with concomitant formation of oxidized glutathione (GSSG). GSHPx plays an important role in the prevention of the deleterious effects of peroxides, harmful intracellular metabolites. generated in the course of tissue metabolism (Zhang et al,1989).

2.6.1.Isoenzymes of Glutathione Peroxidase and other properties

Based on their substrate specifity, two types of GSHPx are generally recognized: selenium-dependent (Droge et al, 1994) and selenium-independent (Eck et al,1989) enzymes. The Se-independent GSHPx activity is attributed to glutathione transferase (GST) isoenzymes alting on a variety of organic hydroperoxides (Paglia et al, 1967). unlike the Se-dependent enzyme which is active with both organic and inorganic peroxides (Ondarza, 1989). Both species are strictly specific for GSH as electron donor ((Monks, 1994).

Reduction by NADPH of the GSSG produced by GSHPx during detoxification of hydroperoxides is catalyzed by glutathione reductase (GSSCRase). a widely distributed flavoprotein (Meister et al, 1994). The reaction catalyzed by GSSGRase is very similar in chemical terms to those catalyzed by lipoamide dehydrogenase, trypanothione reductase and thioredoxin reductase. three closely related flavoprotein reductases which also catalyze the electron transfer between reduced pyridine nucleotides and low or high molecular mass disulfide substrates (Prakash et al, 1988) . In addition to recycling the GSSC produced by GSHPx, GSSGRase has a central role in the intracellular GSII redox status. maintaining very high intracellular GSH/GSSG concentration ratios.

according to the pivotal role of GSH for the optimum situation of the -SH/-SS- intracellular groups (Itzhaki et al, 2000).

GSTs are a multigene familv of ubiquitous dimeric enzvmes which detoxifv noxious organic compounds by conjugating GSH molecules (Slater, 1995). and are implicated also in several endogenous functions (Scholz et al, 1997). In terms of structure function and tissue distribution (Sies et al, 1986). Rat GSTs provide the standard for comparison with GSTs from other organisms and have been grouped into four classes. α , μ , π , θ. According to their physiological, structural and genetic similarities (Seifert et al, 1994) . In addition to using GSH as a common substrate. some GST isoenzymes also display Se-independent GSHPx activity (Vasankari, 1995). This dual activity is explained by the close proximity existing in GSTs between the highly conserved GSH-binding site and the less specific hydrophobic substrate binding area (Warshaw et al, 1985). This allows the nucleophilic attack of GSH leading to adduct formation. characteristic of GST activity and the reduction of organic hydroperoxides, typical of GSHPx activity.

2.7.Glutathione and Glutathine Disulfide

The tripeptide thiol glutathione (GSH) has facile electron-donating capacity, linked to its sulfhydryl (-SH) group. Glutathione is an important water-phase antioxidant and essential cofactor for antioxidant enzymes; it provides protection also for the mitochondria against endogenous oxygen radicals. Its high electron-donating capacity combined with its high intracellular concentration endows GSH with great reducing power, which is used to regulate a complex thiol-exchange system (-SH <---> -S-S-). This functions at all levels of cell activity, from the relatively simple (circulating cysteine/-SH thiols, ascorbate, other small molecules) to the most complex (cellular -SH proteins) (Meister, 1994).

Glutathione is homeostatically controlled, both inside the cell and outside. Enzyme systems synthesize it, utilize it, and regenerate it as per the gamma-glutamyl cycle. Glutathione is most concentrated in the liver (10 mM), where the

"P450 Phase II" enzymes require it to convert fat-soluble substances into water-soluble GSH conjugates, in order to facilitate their excretion. While providing GSH for their specific needs, the liver parenchymal cells export GSH to the outside, where it serves as systemic source of -SH/reducing power. GSH depletion leads to cell death, and has been documented in many degenerative conditions. Mitochondrial (Meister et al, 1994).

GSH depletion may be the ultimate factor determining vulnerability to oxidant attack. Oral ascorbate helps conserve GSH; cysteine is not a safe oral supplement, and of all the oral GSH precursors probably the least flawed and most cost-effective is NAC (N-acetylcysteine). Glutathione (g-glutamylcysteinylglycine, GSH) is a sulfhydryl (-SH) antioxidant, antitoxin, and enzyme cofactor. Glutathione is ubiquitous in animals, plants, and microorganisms, and being water soluble is found mainly in the cell cytosol and other aqueous phases of the living system. Glutathione often attains millimolar levels inside cells, which makes it one of the most highly concentrated intracellular antioxidants.

Glutathione exists in two forms, The antioxidant "reduced glutathione" tripeptide is conventionally called glutathione and abbreviated GSH; the oxidized form is a sulfur-sulfur linked compound, known as glutathione disulfide or GSSG. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress. GSH has potent electron-donating capacity, as indicated by the high negative redox potential of the GSH/GSSH "redox couple" (E'_0 =-0.33v). Its high redox potential renders GSH both a potent antioxidant per se and a convenient cofactor for enzymatic reactions that require readily available electron pairs, the so-called "reducing equivalents." Lewin articulated how a substance with great readiness to donate electrons, when present at high concentrations, has greatly enhanced effectiveness as a reductant. This is reducing power, and is most expressed by GSH where its concentrations are highest (as in the liver). The reducing power of GSH is a measure of its free-radical scavenging, electron-donating, and sulfhydryl-donating capacity. Reducing power is also the key to the multiple

actions of GSH at the molecular, cellular, and tissue levels, and to its effectiveness as a systemic antitoxin.

2.8. Lipid Peroxidation

Lipid peroxidation is a free radical-mediated, chain reaction resulting in the oxidative deterioration of polyunsaturated fatty acids (PUFAs) defined for this purpose as fatty acids that contain more than two double covalent-carbon bonds . Singlet oxygen can produce lipid hydroperoxidase unsaturated lipids by non-radical processes (Pryor and Castle 1984), but the reaction usually requires a radical mechanism (Portar, 1984). Polyunsaturated fatty acids are particularly susceptible to attack by free radicals. Lipid peroxidation is a complex process, ant three distinct phases are recognised: a)initiation b) propagation and c) termination. (Deleve et al, 1990)

Lipid peroxidation are readily oxidised both in vitro and in vivo ,as shown for example by the peroxidized lipids present in age pigments .Molecular oxygen , which as a biradical, contains two unpaired electrons of parallel spin(spin is the quantified angular momentum of the electron which adds to the orbital angular momentum).The parallel spin prevents the direct addition of the electron pair to another molecule and thus chemical bond formation. For a bond to be formed spin reversal has to occur .Since, however , spin reversal takes a long time in comparison with the half-life of activated complexes, molecular oxygen is a relatively weak oxidant ,and autooxidation of lipids in living organisms is a slow process . Nevertheless, if lipids are activated to free radicals R via removal of hydrogen mediated by a free radical initiator ,they enter into reaction with molecular oxygen more readily. A peroxy free radical ROO is formed during the reaction ,and this process is called lipit peroxidation (LPO). Polyunsaturated fatty acids(PUFA) are especially liable to peroxidation since the C-H bond of their so-called a-methylene carbon atom next to the double bond is weak, and thus they may be regarded as partially activated. Therefore , as the initial step of LPO ,hydrogen is removed from this carbon atom (the hydrogen of the a-

methylene carbon atom is called allylic hydrogen) (Del Maestro 1980, Pryor,1973).

1.During initiation a free radical substance (X) reacts with a fatty acids chain containing one or more multible bonds.

2. A radical centre is formed on the carbon atom next to the double bond (a-methylene carbon). After the free radical initiator X has abstracted a hydrogen thereby ceased to be a free radical (XH). At the same time, an alkyl radical R is formed from the lipid. B) Several configurational changes follow the initiation almost immediately- massive electron transfers occur (Yagi et al, 1982).

i)transfer of the free electron to other C atoms; ii)this moves the double bonds closer, leading to the formation of conjugated double bonds, which can be demonstrated by spectrophotometry as signs of early radical damage; iii)some double bonds adopt the trans configuration, instead of the cis configuration characteristic of fatty acids.

3.The oxygen is added to the alkyl radical and a peroxy radical ROO is formed.

4.Peroxy radical abstract hydrogen from the nearby moleculs. (RH, which may be other unsaturated fatty acids, proteins, antioxidants or nucleic acids) and thus metastable lipid hydroperoxides ROOH are formed; these products are transiently stable (Rudneva, 1999).

5. 6-hydroperoxide is capable of spontaneous decomposition to OH and alkoxy radicals RO. As a result of metal catalysis alkoxy and peroxy radicals and hydroxyl or hydrogen ions are formed and the fatty acid is decomposed in to aldehydes and alkyl radical fragments.

6. Aklyl radicals may react with surrounding radicals, e.g. alkoxy radical, and thus the process is terminated as a result of the formation of O-bonded bridges between the molecules or C-C bonds may be formed with another alkyl radical.

Initiation may thus start a chain reaction which, if uncontrolled by defence mechanisms, may lead to extensive damage of the surrounding molecules. Autooxidation of lipids eventually leads to the formation of new C-C bonds. Since lipids are membrane constituents, it is easy to sea to what extent cross-linking may damage the membranes (Sasaki et al, 1988; Kehrer et al, 1994)).

Autooxidation is considerably accelerated by the prences of transition metals (iron, copper) enhancing the degradation of metastable hydroperoxides. Lipids, which are readly oxidised, promote the metal-catalysed oxiditation, in the presence of O_2, of more inert cellular components like proteins or nucleic acids. Autooxidation is a very slow, circumscribed process under physiological conditions. Under pathological conditions, however, it is accelerated, becomes more extensive and may damage all basic biomolecules. The metalstable lipid peroxides formed during LPO, malondialdehyde (MDA), and other stable end products of LPO, e.g. 2-alkenals and 4-hydroxyl-2-alkenals, may reach more distant parts of the cell or other cells and tissues owing to their relative stability. Thus LPO may also damage cells or tissues which are nor directly exposed to peroxidative damage (Roels et al, 1982).

Chapter Three

MATERIAL AND METHODS

3.1.Materials and Chemicals

Experiments were conducted for two different amphibian species: R.r.ridibunda and B.viridis. They were collected around Salihli (Kurşunlu)/Turkey from their natural habitat and staged according to the criteria of Taylor and Kollros and fed with bolied spinach. For enzyme analysis; larvae and tissues were stored at –20 ^{0}C, until used. They were thawed at +4 ^{0} C then 2,6 g larval or tissue samples were homogenised in pre-cooled 20 mM potassium phosphate buffer, pH 7.4 (10mL) and centrifuged 15000 rpm for 15 min. For LPO determination: Tissue sample is weighed and chilled in ice-cold 1.15% KCl, 10% (w/v) homogenate is prepared using a homogeniser.

The experiments were performed using 6-Hydroxydopamine (2,4,5 trihydroxyphenyl ethylamine), commassie brillant blue G and P, ethylene diamine tetraaceticacid (EDTA), Sodium dodecyl sulfate (SDS), bromo phenol blue) amoniumpersulphate (APS), phenylmethylsulfonyl floride (PMSF), N,N,N' ,N'-tetramethylethylenediamine (TEMBD), dithiothicitol (DTT), carboxymethyl-cellulose, bovine serum albumin, ovalbumin, lactoglobulin, trypsinogen, lysozyme, cytochrome c, carbonic anhydrase, aprotinin, blue dextran, polyethylene glycol (PEG)-2000; 3350; 5000 (Sigma St.Louis, USA), 13-mercaptoethanol, acrylamide, N,N'methylenebisacrylamide, glycerol, tris-base, H_3B0_{33} Folin Ciocalteu's Reagent, NaOH, glutaraldehyde, 11202, (Merck, Darmstadt, Germany), KCI, NaCI, KH2P04, K_2HP0_4, QIIt)2S04, methanol (Riedel de Haen, Germany). All chemicals were used analytically pure.

Optical measurements were made up by a spectrophotometer (UV-1601, Shimadzu, Japan) with thermocouple system. Also, thermostat (Lauda, Germany), cryostat (Heto-therm, Germany), homogenizator (ICA, Germany), pH-meter (Schon, Germany), peristaltic pump (Multi-Mix, Germany), electrophoresis (BIO-RAD, Protean XII, USA), atomic absorption spectrophotometer (Varian, SpectrAA-300 plus, Austria), refrigerated centrifuge (Hettich Universal 1 6R, Germany), vortex (Nuve) were used at the studies of experimental.

3.2.Experiments

3.2.1 Superoxide Dismutase Activity Assay

All solutions used in this study were made up with bidistilled water, according to the following scheme,

Phosphate Buffer 0.1M, pH 5.5-8.0

2.774 g K_2HPO4 and 1.7025 g KH_2P0_4 were dissolved in 250m1 of water and pH was adjusted with 0.1M NaOH solution.

Potassium Pyrogallate Solution

50 g KOH was dissolved in 100 ml of water and sparged with N_2. Then 5 g apilol was added into this solution. The pyrogallate solution was used as oxygen orbent in the N_2 gases system. 1.028 mg 6-OHDA was dissolved in 1 ml of KCl solution (1 mM, pH 2.00) which was prepared before used and sparged with nitrogen. During the experiments stock solution was reserved in N_2 gas environment which was passed through the pyrogallate solution at $+4^0$C.

Definition of Unit of Superoxide Dismutase Activity in the Standard Activity Assay Condition

Simple assay system for SOD based on the inhibitory effects of SOD on the spontaneous autoxidation of 6-hydroxydopamine (Crosti et al. 1987).

Autoxidation rate of 6-OHDA (4x 10^{-4} M) in 0.1 M phosphate buffer pH 7.4 which was saturated by air-0_2 (8.2 mg/I) was determined by observing of the absorbance changes at 490 nm in 15 sec time intervals at 25^0C. SOD activity

assays were carried out under the same conditions by adding solution required to drop the half of the initial absorbance value of 6-OHDA adanon at the 90 sec. 1 'U is the amount of SOD required to inhibit the initial rate of 6-tydoparnine autoxidation by 50%.

3.2.2. Catalase Activity Assay

Gregory and Fridovich (1974) report on a sensitive activity stain for catalase applicable to a polyacrylamide gel electrophoretogram, Haining and Legan (1972) describe a polarographic assay utilizable in tissue homogenates, and Kroll et al. (1989) discuss a rapid method for estimating the bacterial content of foods.

There are numerous assays for catalase. The subject has been reviewed by Maehly and Chance (1954) and Chance and Maehly (1955). The assay used at Worthington follows:

Method: Essentially that described by Aebi (1967) in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. One unit decomposes one micromole of H_2O_2 per minute at 25 C and pH 7.0 under the specified conditions.

Reagents

- 0.05 M Potassium phosphate, pH 7.0
- 0.059 M Hydrogen peroxide (Merck's Superoxol or equivalent grade) in 0.05 M potassium phosphate, pH 7.0

Enzyme

Immediately prior to use dilute the enzyme in 0.05 M phosphate buffer, pH 7.0 to obtain a rate of 0.03-0.07 □A/min.

Incubate in spectrophotometer for 4-5 minutes to achieve temperature equilibration and to establish blank rate if any. Add 0.1 ml of diluted enzyme and record decrease in absorbance at 240 nm for 2-3 minutes. Calculate ΔA_{240}/min from the initial (45 second) linear portion of the curve.

3.2.3.Glutathione Peroxidase Assay

Glutathione Peroxidase activity was measured in a coupled system by measuring the decrease of NADPH at 340 nm. The reaction mixture (1.060 mL) contained in order of addition 1.0 mL Reagent contained glutathione 4 mmol/l, Glutathione reductase >0.5 U/L, NADPH 0.28 mmol/L and 50 mM phosphate buffer pH 7.2 then 20 µL diluted sample is added finally 40 µL cumene hydroperoxide is added and vigorously mixed. After one minute, initial absorbance of sample and reagent blank are read and timer is started, after one and two minutes, absorbances read again and reagent blank value is substracted from that of the sample (Paglia et al, 1967).

3.2.4. Lipid Peroxidation Assay

Peroxidized lipids occurring in animal tissues have been generally recognized to affect several cardiovascular, pulmonary, or hepatic diseases and to be a principal cause of aging. Lipid peroxidation progresses absorbing molecular oxygen into activated free radicals produced from labile polyunsaturation in fatty acid chain. The estimation and determination of the peroxidation have been carried out by measurement of oxygen uptake, photometric determination of coniugated diene at 230 nm, thiosulfate titration of iodine liberated from potassium iodide by peroxide oxidation, and colorimetric determination of a thiobarbituric acid (TBA) reactive substance at 535 nm. The method applicable to animal tissue was limited to TBA reaction because of its simplicity in operation and high sensitivity

During the past several decades numerous procedures have been introduced for the measurement of lipohydroperoxide or malonaldehyde precursor , using TBA. A process for coloration of heating the assay sample with TBA in acidic solution is common to all these procedures, but in other respects each operation is different. Deproteinized supernatant fluid with or without an appropriate preceding incubation of tissue homogenate is used as an assay sample in one

procedure , while another procedure recommends employing the precipitate obtained by adding some protein precipitants to the tissue homogenate and others prefer to use acidified whole homogenate in the assay procedures for the test eventually yield different assay results. Therefore, the data from the different investigators did not allow a direct comparison.

In this paper, we describe a conventional and reproducible procedure for analyzing TBA-reactive substances in the homogenates of various organs. The dependency of color intensity on acidity and amount of medium and other technical modifications are presented.

Materials And Methods

Reagents. Thiobarbituric acid was obtained from Wako Pure Chemical Industries, Ltd. and recrystallized twice with water. Phosphoric acid, n-butanol, and trichloroacetic acid (TCA) were of reagent grade. Malonaldehyde was prepared by the hydrolysis of 1,1,3,3-tetraethoxy-propane

Apparatus. A Hitachi Model 556 double-wavelength double-beam spectrophotometer was used. Tissues were removed and homogenized with cold 1.15% KCI to make a 10% homogenate. To a 0.5 ml of 10% homogenate pipetted into a 10-mI centrifuging tube were added 3 ml of 1% phosphoric acid and 1 ml of 0.6% TBA aqueous solution. The mixture was heated for 45 mm on a boiling water bath. After cooling, 4 ml of n-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation and absorbance was measured at 535 and 520 nm. The difference was used as the TBA value. When the dependency of color development on acid concentration or pH was tested, the above procedure was modified by eniploying 3 ml each of 20, 10, 5, 0.5, and 0.1% TCA or 0.1 N HCl-citrate buffer instead of 1% phosphoric acid (Okhawa et al, 1979).

3.2.5 Protein Assay Method

Protein concentrations were estimated with the coomassie blue dye-binding assay according to the method of Bradford, 1976

Coomassie Brillant Blue Solution

Coomassie Brillant Blue 0-250 (lOOmg) was dissolved in 50 ml 95% ethanol. To this solution, 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to final volume of I liter. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brillant Blue 0-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid.

Stock Standard Protein Solution

10 mg BSA (crystalline) was diluted in to 100 ml with 0.02 M phosphate buffer, pH7.4.

Protein solutions containing 1.2 to 6 ug protein in a volume up to 0.1 ml were pipetted in to test tubes. One milliliter of protein reagent was added to the test tube and the contents were mixed. Absorbance at 595 nm was measured after two minutes.

In 1 ml cuvettes against a reagent blank prepared from 0.1 ml of buffer solution in I ml assay volume. The amounts of protein were plotted against the corresponding

Chapter Four
RESULTS

4.1 The Activities of Antioxidant Enzymes and Lipid Peroxidation levels in the Tadpoles R.r.ridibunda and B.viridis dependent on the development period.

The variations of antioxidant enzyme activities and LPO levels were investigated during the metamorphosis period of B.viridis and R.r.ridibunda tadpoles.

As can be seen from Fig 4.1, SOD activity variations were observed as gradual increases dependent on time in B.viridis from 0.25 ± 0.013 to 2.6 ± 0.13 IU/mg between 1-8 weeks. Although there was no drastic change between 1-5 weeks in tadpoles of R.r.ridibunda, after week 5, a sharp increase ($p<0.001$) was determined from 0.4 ± 0.02 to 2.3 ± 0.12 IU/mg .

Figure 4.2 shows that, CAT activity values were not significantly different up to the 3rd week in both species. CAT activity increased sharply in B.viridis after the 3rd week, but it increased slightly after the 5th week in R.r.ridibunda. CAT activity values in B.viridis and R.r.ridibunda which were very similar at the first week, reached 201 ± 10.05 and 49 ± 2.45 IU/mg at the 8th week, respectively.

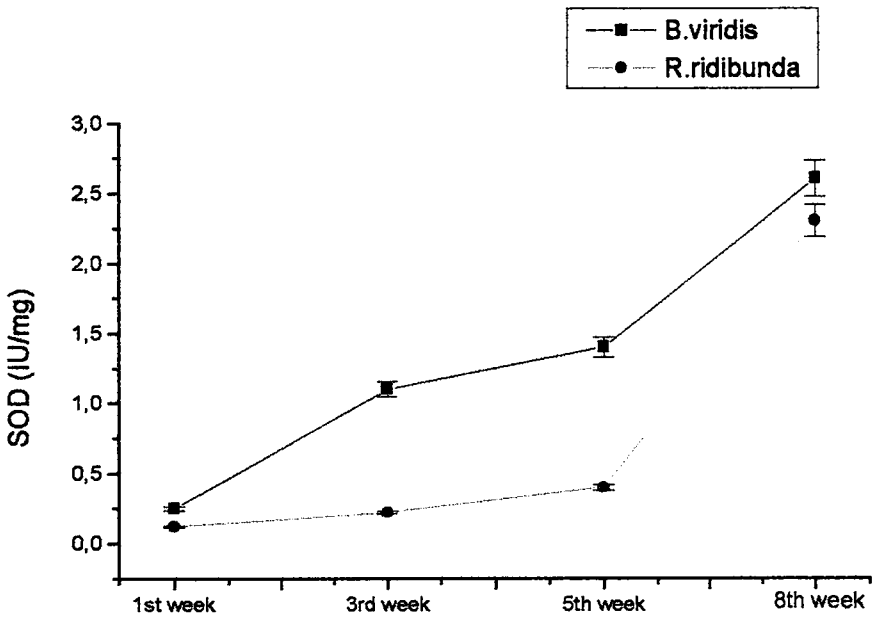

Figure 4.1 SOD Activity variation for development period of R.r.ridibunda and B.viridis tadpoles. Values given are the means of at least three experiments.

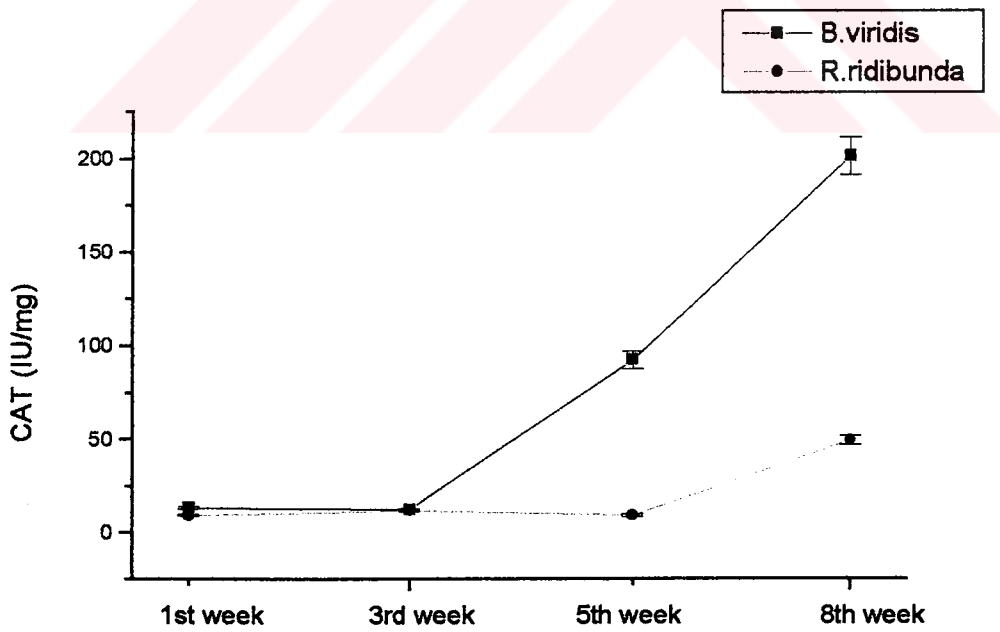

Figure 4.2 CAT activity variations for development period of B.viridis and R.ridibunda tadpoles. Values given are the means of the at least three experiments

According to Figure 4.3, GSH-Px activity significantly ($p<0.001$) increased in the tadpoles of R.r.ridibunda and B.viridis from 0.25 ± 0.045 to 3.45 ± 0,21 and from 0.89 ± 0.125 to 4.1 ± 0.21 IU/mg, respectively. While variations of the GSH-Px activity of R.r.ridibunda tadpoles showed the gradual increases during the development period, it slightly increased up to 5th week then sharply increased in the B.viridis ($p<0.001$).

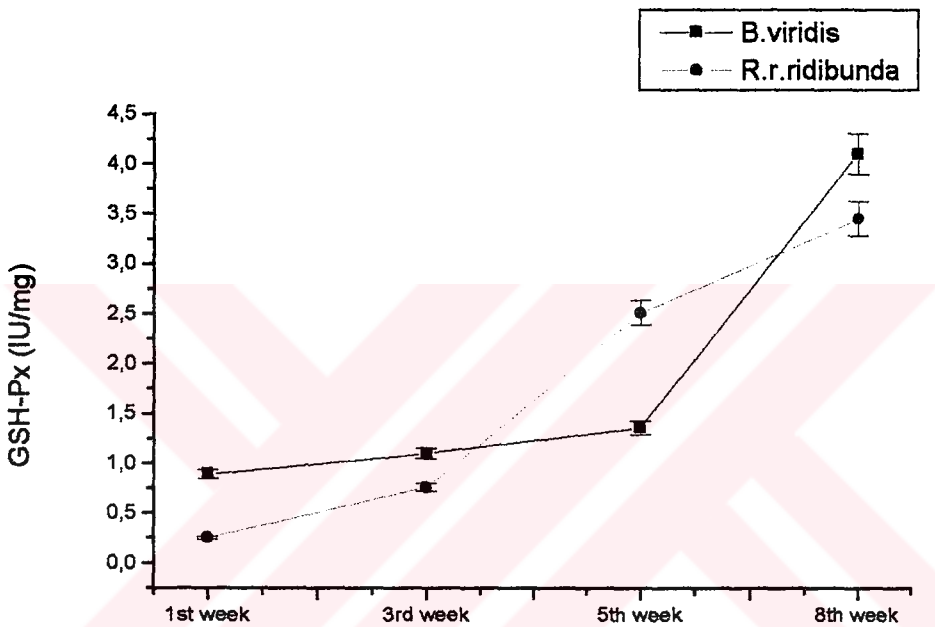

Figure 4.3 Glutathione Peroxidase activity variations during development period of *B.viridis* and *R.r.ridibunda*.Values given are the means of the at least three experiments.

As can be seen from Figure 4.6 , while LPO levels decreased slightly from 206 ± 10.3 to 146 ± 7.3 nmol MDA/g tissue in R.r.ridibunda, a sharp fall was ($p<0.001$) observed in B.viridis tadpoles from 198 ± 9.9 to 23 ± 7.3 nmol MDA/g tissue during the development period.

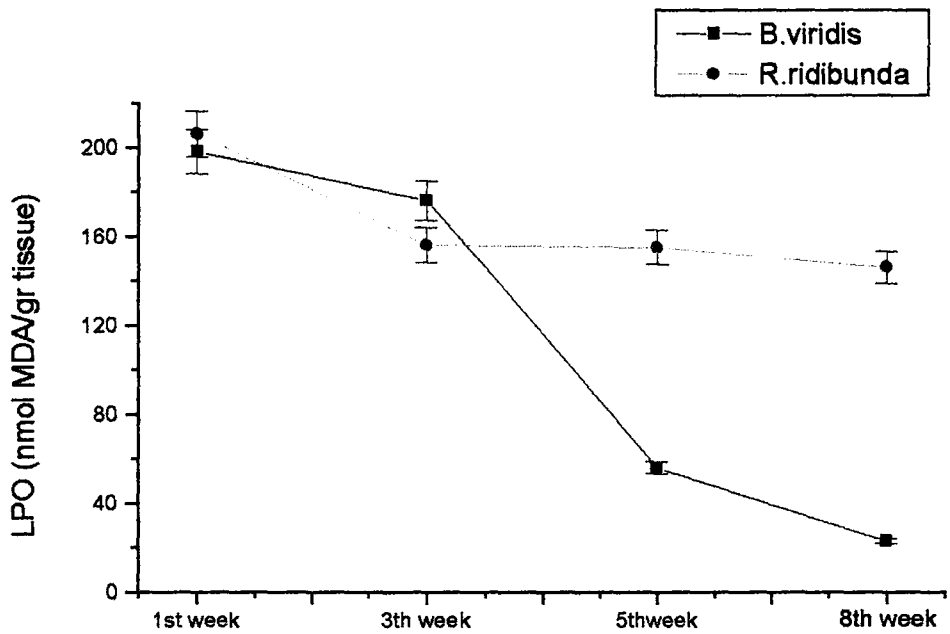

Figure 4.6 LPO levels for development period of B.viridis and R.r.ridibunda. Values given are the means of the at least three experiments.

4.2 The Activities of Antioxidant Enzymes, and Lipid Peroxidation Levels in the Tissues of young R.r.ridibunda and B.viridis dependent on the active and hibernation periods.

The activities of SOD, CAT, GSH-Px and were investigated in the five vital tissues of R.r.ridibunda and B.viridis dependent on their active and hibernation period.

As can be seen in Figure 4.6 and 7, SOD activities in liver were significantly ($p<0.05$) higher when compared with the other tissues. SOD activity of liver in R.r.ridibunda and B.viridis in the active and hibernation period were 15 ± 0.95 and 42 ± 2.3 ; 9.1 ± 1.7 and 26 ± 1.66 IU/mg, respectively. SOD activity in liver of B.viridis.

According to Figure 4.8 and 4.9, CAT activities of predominantly terrestrial amphibian B.viridis were also higher than aquatic amphibian R.r.ridibunda as well as the active period. Maximum CAT activity were observed again liver in B.viridis and R.r.ridibunda in the active and hibernation period as 2001 ± 103.1 ; 1692 ± 78.9 and 1603 ± 63.4 ; 1391 ± 56.9 IU/mg, respectively. Minimum CAT activities again were observed in packed erythrocytes.

According to results obtained from Figure 4.10 and 4.11, GSH-Px acitivities in the tissues of young R.r.ridibunda were lower than in B.viridis except that heart. Maximum GSH-Px activities were observed in heart of B.viridis in the active season as 121 ± 2,6 IU/mg.

As can be seen in Figure 4.12 and 4.13, LPO levels of liver of R.r.ridibunda (active and hibernation period) markedly ($p<0.001$) different from other tissues. In contrast to highest antioxidant enzyme activities observed in the tissues of B.viridis, LPO levels were lower than in R.r.ridibunda apart from lung. While minimum LPO levels were observed in heart of B.viridis, liver of R.r.ridibunda showed the highest LPO levels when the compared with the other tissues during the hibernation period. Minimum LPO levels observed in lung of R.r.ridibunda and erythrocytes of B.viridis. When the seasons were concerned, the LPO levels relatively lower in hibernation period because of lower oxygen demand as well as the old specimens.

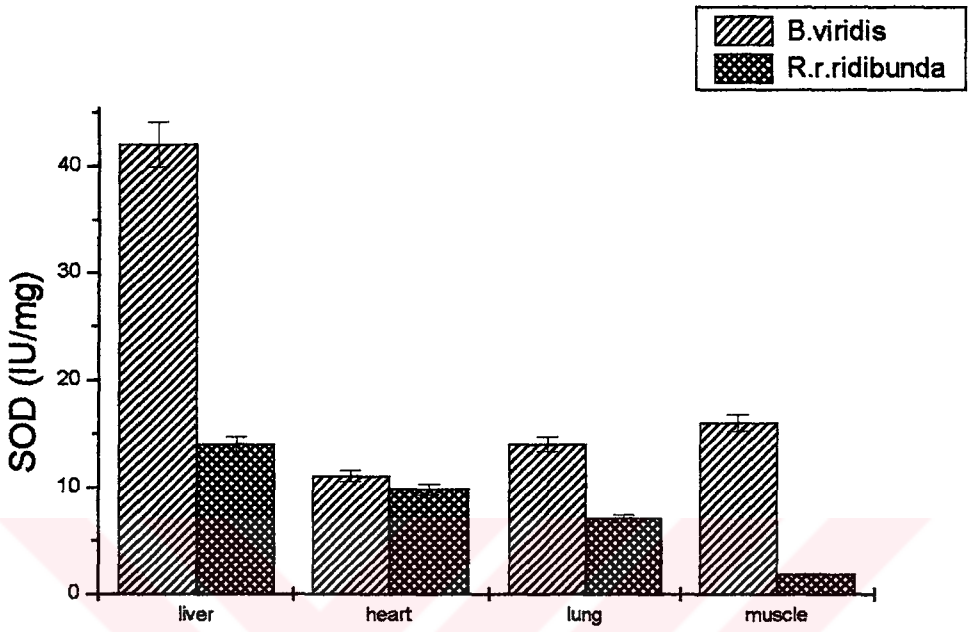

Figure 4.6 SOD activity variations in the tissues of young B.viridis and R.r.ridibunda in the active period. Values given are the means of three separate experiments.

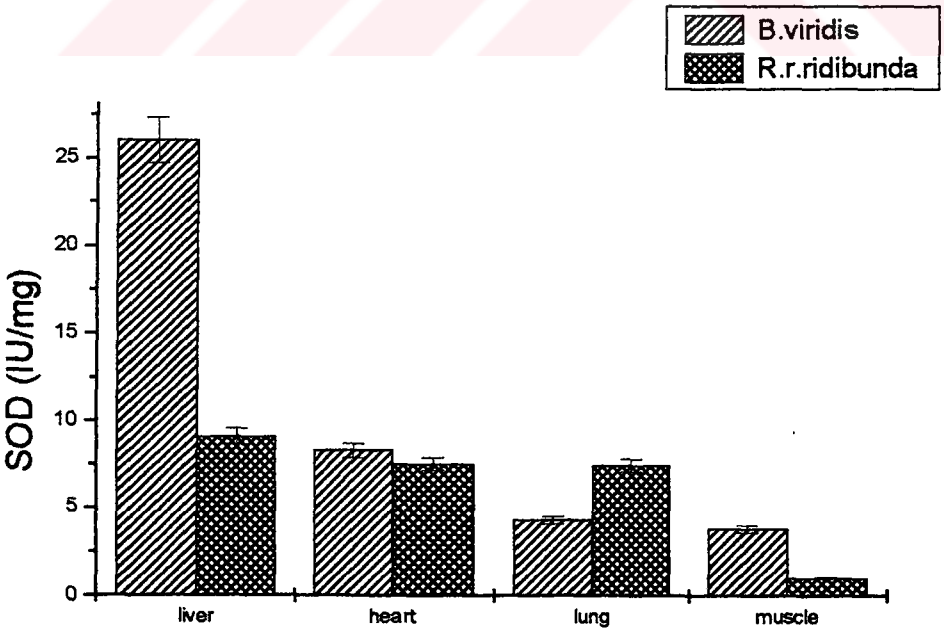

Figure 4.7 SOD activity variations in the tissues of young B.viridis and R.r.ridibunda in the hibernation period.Values given are the means of three separate experiments.

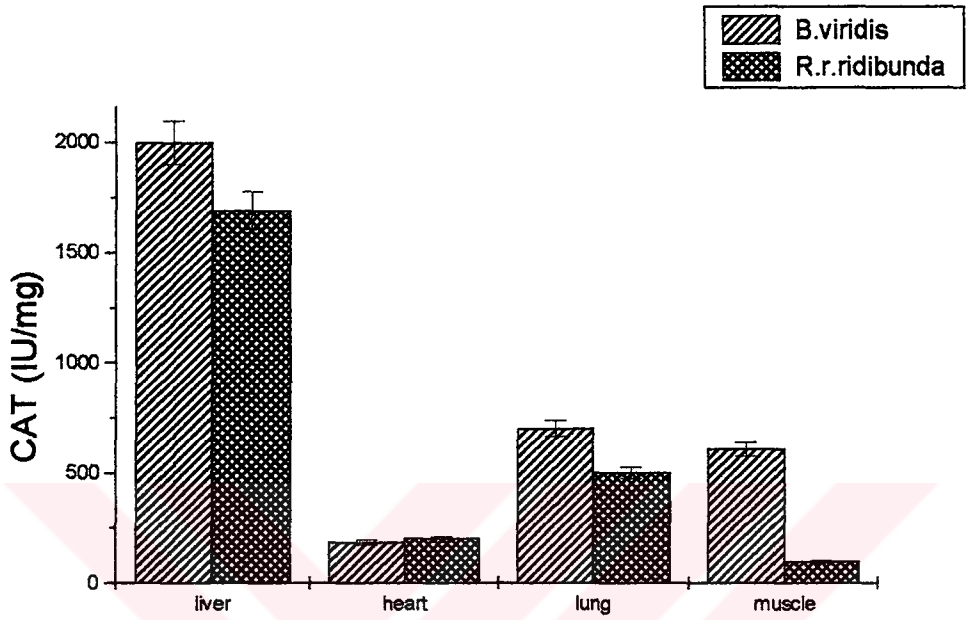

Figure 4.8 CAT activity variations in the tissues of young B.viridis and R.r.ridibunda in the active period.Values given are the means of three separate experiments.

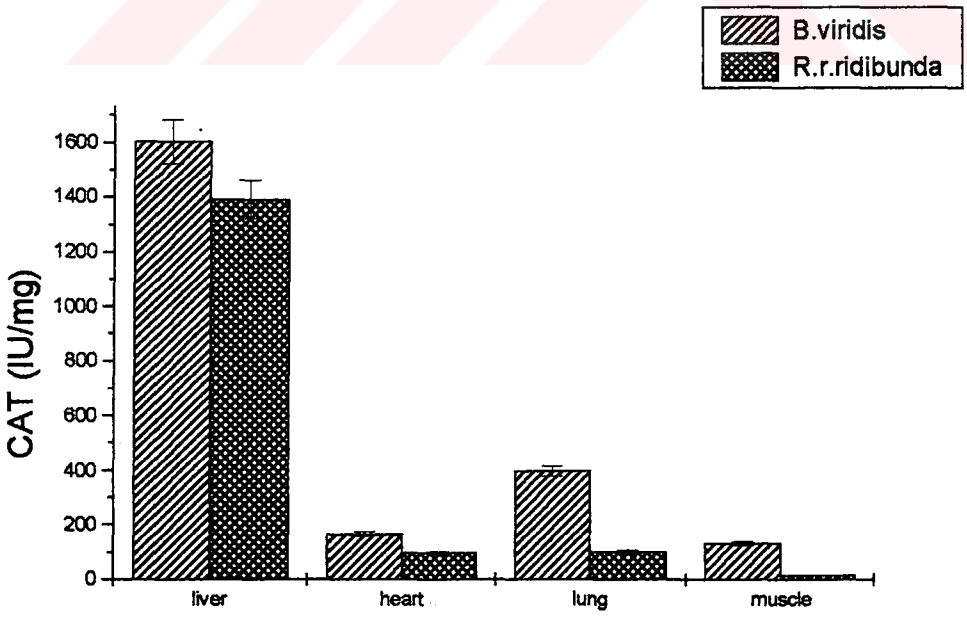

Figure 4.9 CAT activity variations in the tissues of young B.viridis and R.r.ridibunda in the hibernation period. Values given are the means of three separate experiments.

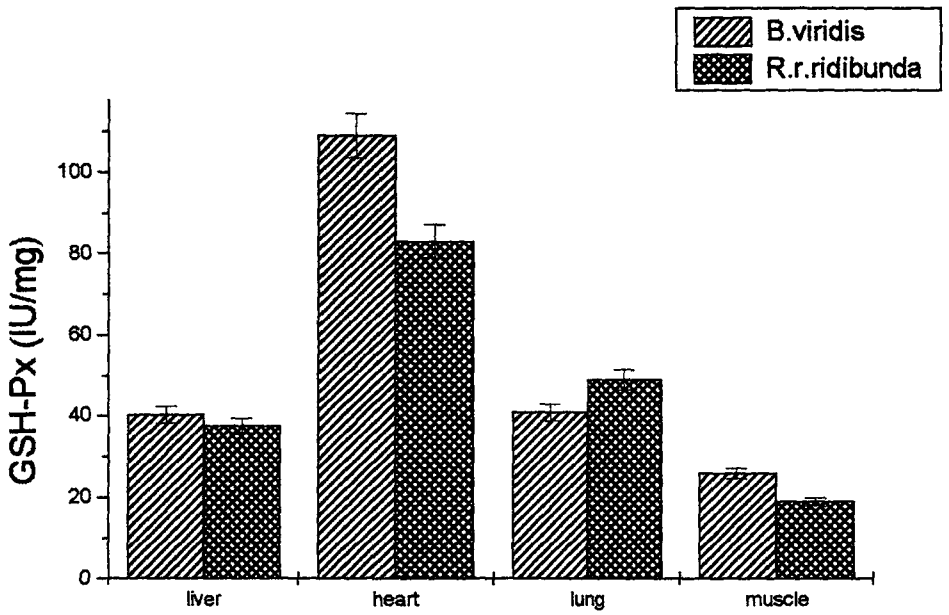

Figure 4.10 GSH-Px activity variations in the tissues of young B.viridis and R.r.ridibunda in the active period. Values given are the means three separate experiments.

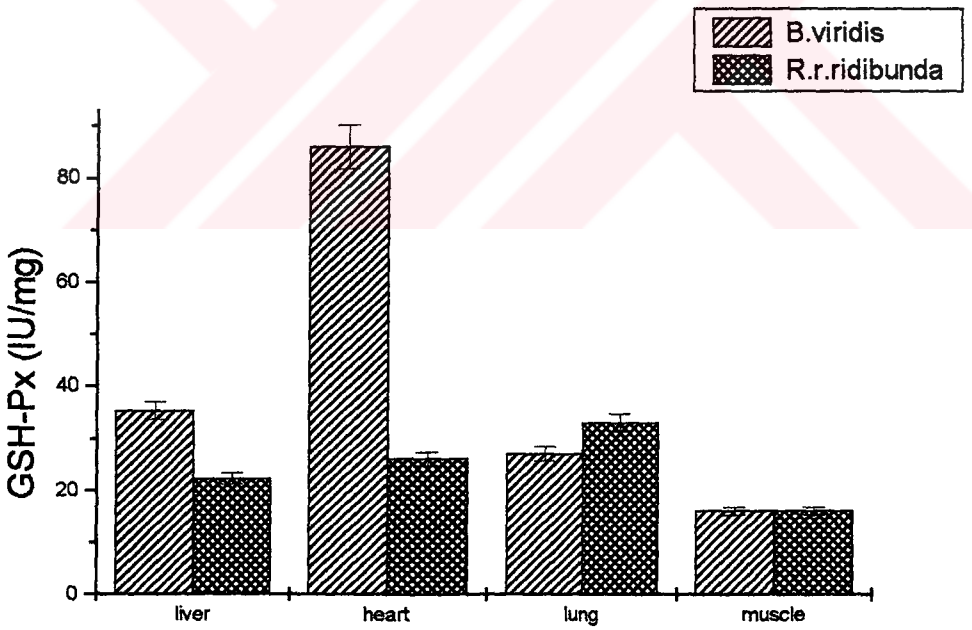

Figure 4.11 GSH-Px activity variations in the tissues of young B.viridis and R.r.ridibunda in the hibernation period. Values given are the means of three separate experiments.

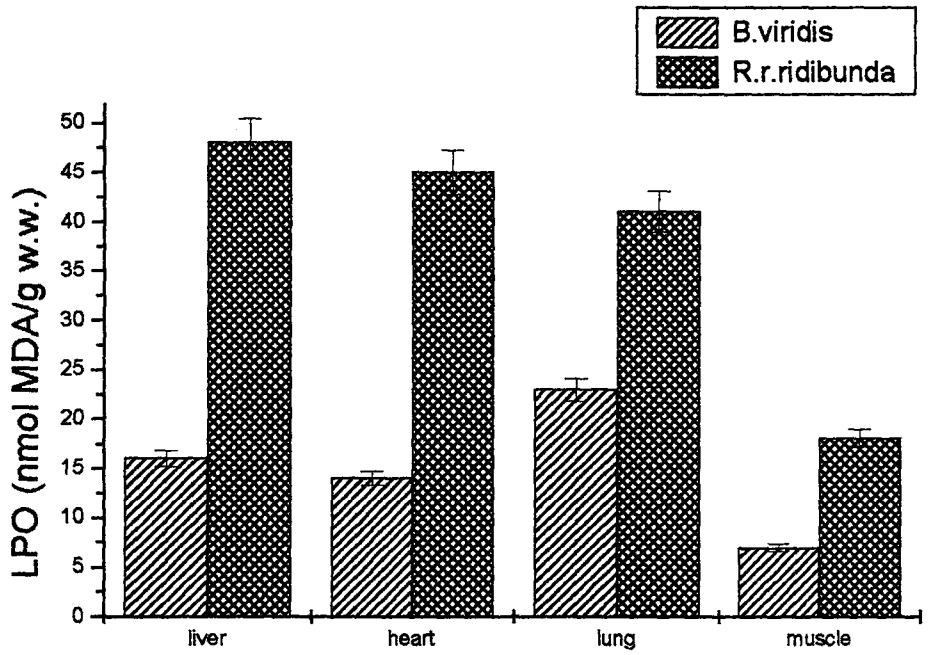

Figure 4.12 LPO levels in the tissues of B.viridis and R.r.ridibunda in the active period. Values given are the means of three separate experiments.

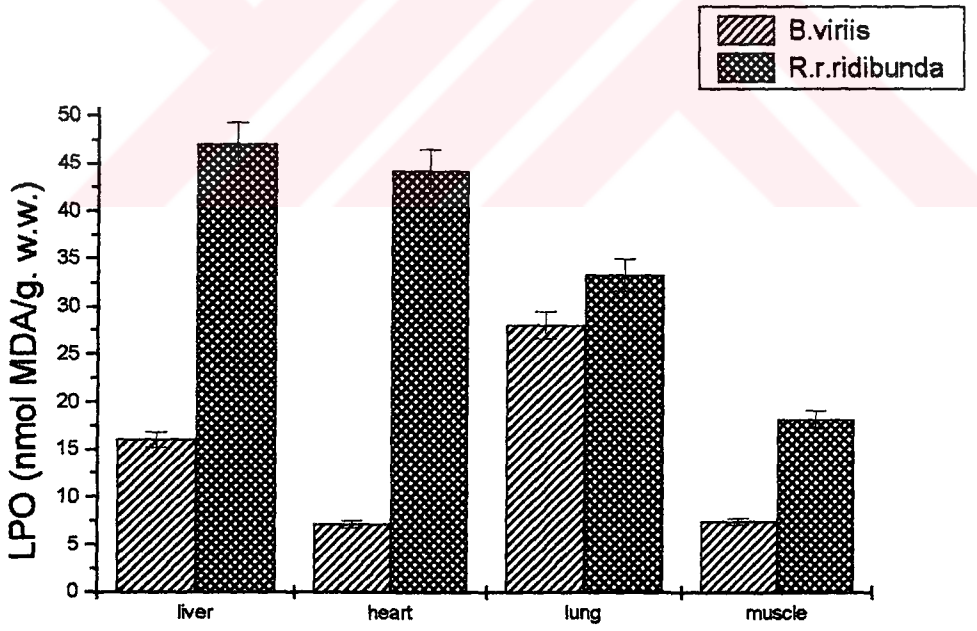

Figure 4.13 LPO levels in the tissues of young B.viridis and R.r.ridibunda in the hibernation period. Values given are the means of three separate experiments.

4.3 The Activities of Antioxidant Enzymes and Lipid Peroxidation in the Tissues of old R.r.ridibunda and B.viridis dependent on the active and hibernation periods.

The activities of SOD, CAT, GSH-Px and LPO levels were investigated in the five vital tissues of R.r.ridibunda and B.viridis dependent on their active and hibernation period.

As can be seen in Figur 4.14 and 4.15 SOD activities in liver were significantly ($p<0.05$) higher when compared with the other tissues. SOD activity of liver in R.r.ridibunda and B.viridis in the active and hibernation period were 16.9 ± 0.85 and 46 ± 2.3 ; 33 ± 1.7 and 10.7 ± 0.56 IU/mg, respectively. SOD activity in liver of B.viridis.

Figure 4.16 and 4.17 shows that CAT activities of predominantly terrestrial amphibian B.viridis were higher than aquatic amphibian R.r.ridibunda. Maximum CAT activity were observed again liver in B.viridis and R.r.ridibunda in the active and hibernation period as 2631 ± 130.1 ; 1795 ± 89.9 and 1898 ± 93.4 ; 1602 ± 78.9 IU/mg, respectively. Minimum CAT activities were observed in packed erythrocytes.

As can be seen in Figure 4.18 and 4.19 , LPO levels of liver of R.r.ridibunda (active period) markedly ($p<0.001$) different from other tissues. In contrast to highest antioxidant enzyme activities observed in the tissues of B.viridis, LPO levels were lower than in R.r.ridibunda apart from lung. While maximum LPO levels were observed in liver of R.r.ridibunda, lung of B.viridis showed the highest LPO levels when the compared with the other tissues. Minimum LPO levels observed in lung of R.r.ridibunda and erythrocytes of B.viridis. When the seasons were concerned, the LPO levels relatively loer in hibernation period because of lower oxygen demand.

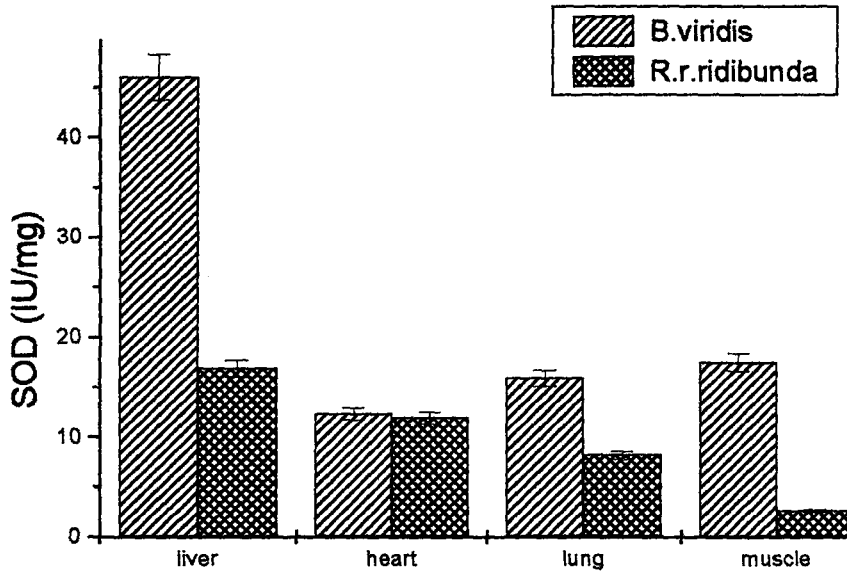

Figure 4.14 SOD activity variation in the tissues of *B.viridis* and *R.r.ridibunda*
Values given are the means of at least three experiments.

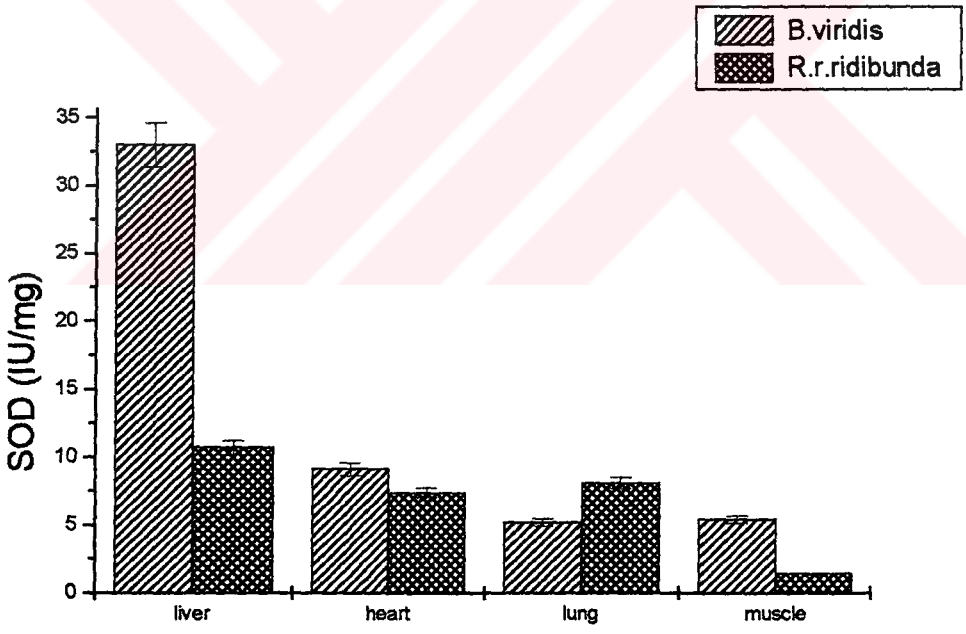

Figure 4.15 SOD activity variations in the tissues of R.r.ridibunda and B.viridis in the hibernation period.Values given are the means of three separate experiments.

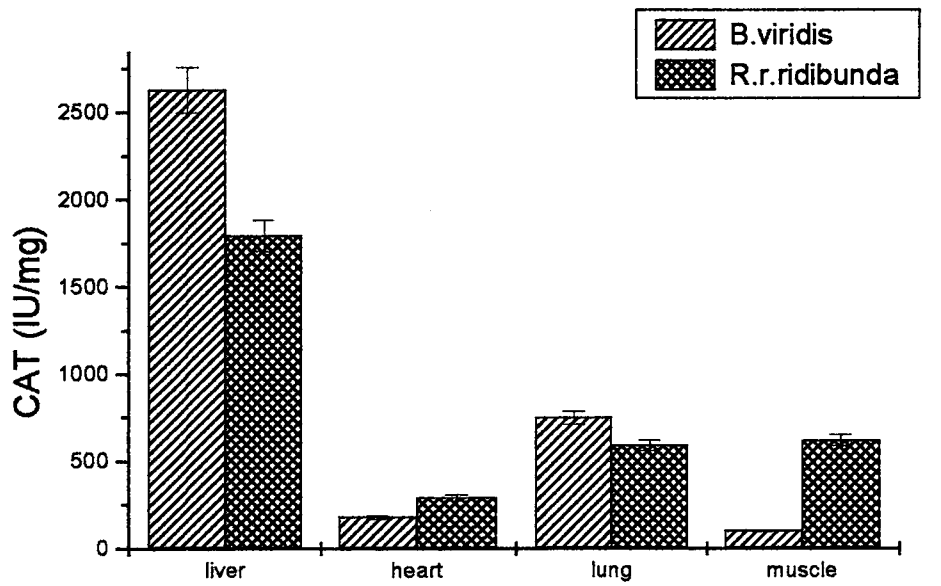

Figure 4.16 CAT activity variations in the various tissues of *B.viridis* and *R.r.ridibunda*
Values given are the means of at least three experiments.

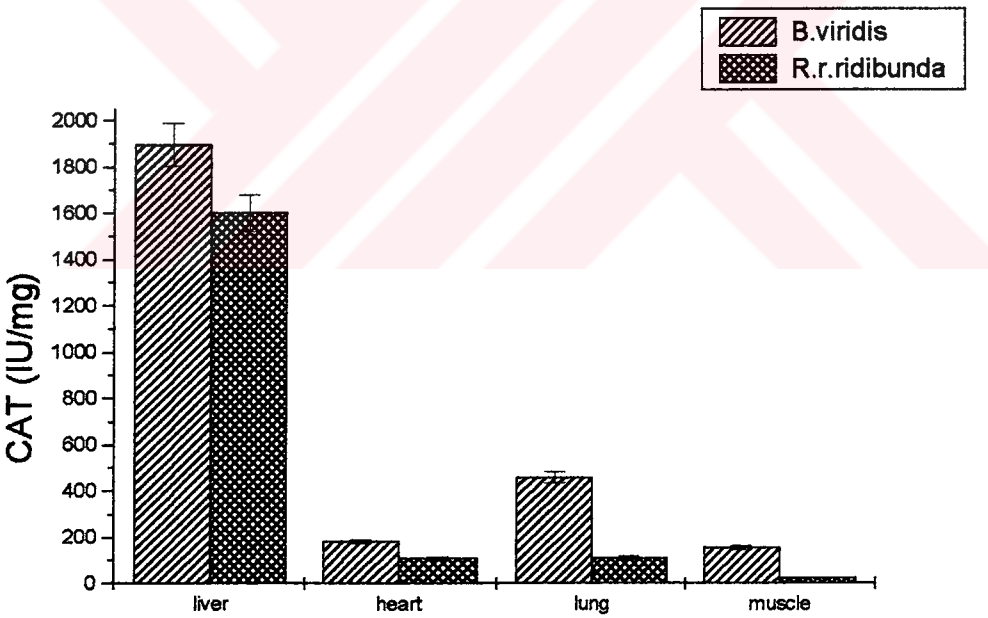

Figure 4.17 CAT activity variations in the tissues of R.r.ridibunda and B.viridis in the hibernation period.Values given are the means of three separate experiments.

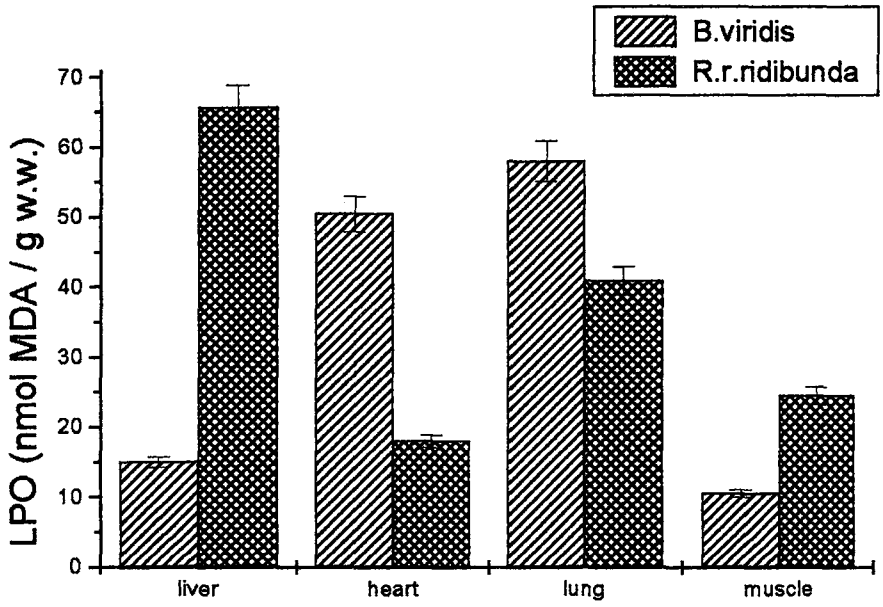

Figure 4.18 LPO levels variations in the various tissues of *B.viridis* and *R.r.ridibunda*. Values given are the means of the at least three experiments.

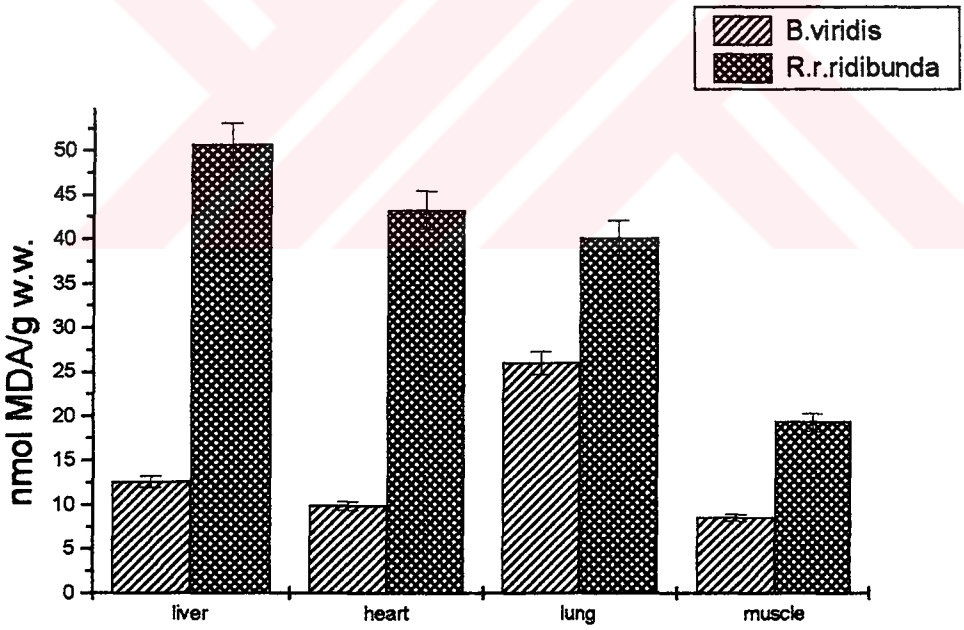

Figure 4.19 LPO levels in the tissues of R.r.ridibunda and B.viridisin the hibernation period. Values given are the means of three separate experiments.

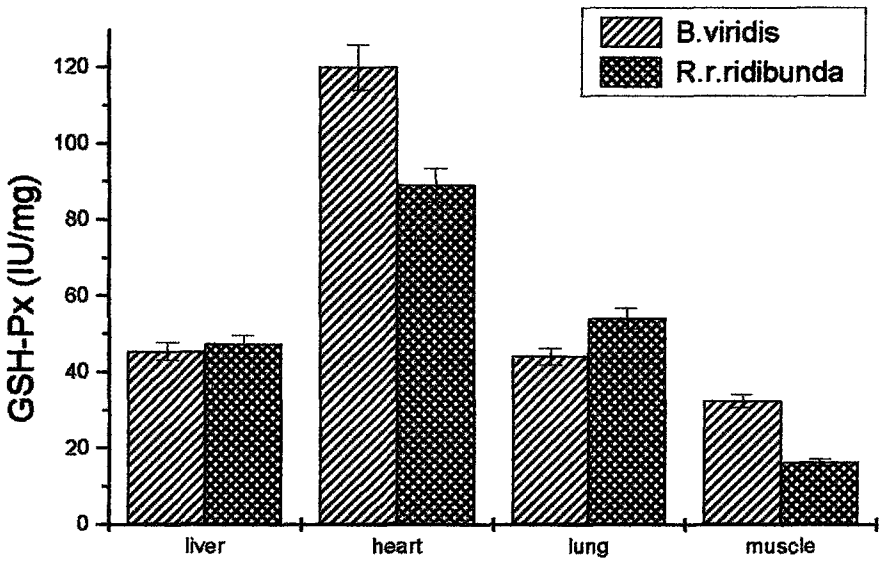

Figure 4.20 GSH-Px activity variations in the various tissues of *B.viridis* and *R.r.ridibunda*. Values given are the means of the at least three experiments.

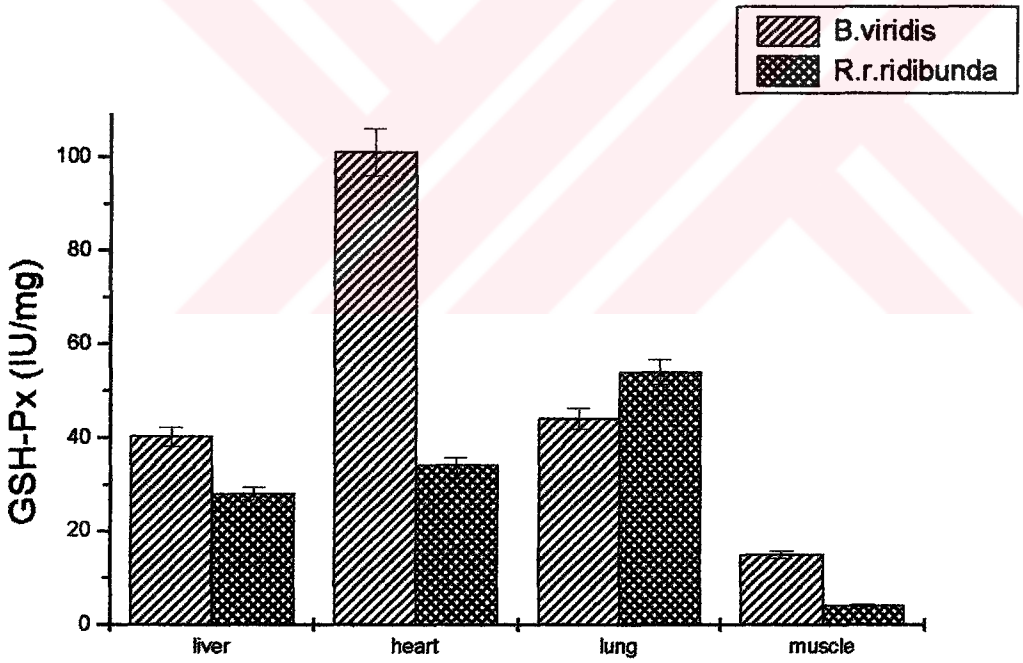

Figure 4.21 GSH-Px activity variations in the tissues of R.r.ridibunda and B.viridis in the hibernation period. Values given are the means of three separate experiments.

Tablo 4.1 Erythrocyte Antioxidant Enzyme Activities and Lipid peroxidation Levels in R.r.ridibunda and B.viridis

	SOD	CAT	GSH-Px	LPO
Young R.r.ridibunda Active period	0.40	3.1	0.86	47
Young B.viridis Active Period	1,01	6	6	19
Young R.r.ridibunda Hibernation period	0.37	2.1	3.9	36
Young B.viridis Hibernation Period	0.89	3.2	5.4	12.3
R.r.ridibunda Active period	0.26	5.9	1	42
B.viridis Active Period	2.31	8	7	9.8
R.r.ridibunda Hibernation period	0.26	2.3	4.9	35.5
B.viridis Hibernation Period	1.31	4.1	5.7	15.3

CONCLUSIONS

Antioxidant enzymes; SOD GSH-Px and CAT in vivo play important roles in removal of damaging effects caused by reactive oxygen species. While antioxidant enzyme activities increased with different percentages, LPO levels decreased during their 8 weeks development period in B.viridis and R.r.ridibunda tadpoles. However, TSA levels were relatively correlated with LPO levels with respect to antioxidant enzyme activities.

SOD activity increased 10.4 fold in B.viridis and 19.2 fold in R.r.ridibunda between 1-8 weeks. According to a report by Rudneva et al , SOD activities of larvae of N.melanostomus and P.marmoratus increased 6,5-fold during their development .

The activities of CAT, another important radical scavenger, were not statistically different among the species at the end of the 1st week, however they reached 201 ± 10.05 and 49 ± 2.45 in B.viridis and R.r.ridibunda respectively at the end of the 8th week. These data may be as a result of increasing SOD activity causing over-production of H_2O_2 during the developmental period. It has been reported that hydrogen peroxide locates on the plasma membrane at the sites of contact between macrophages and adjoining cells in the tails of metamorphosing R.japonica tadpoles.

In our investigation, we found increased SOD and CAT activities during the development period in R.r.ridibunda and B.viridis tadpoles and similar reports were also observed for other animals during early development such as fruit fly

C.capitata , the insect H.zea , fish eggs of N.melanostomus and B.sanguinolentus , Xenopus and guine a pig. Increases of antioxidant enzyme activities must reflect the gene expression during development and synthesis of these proteins. SOD and CAT activities, which play an important role against ROS, and suppress the oxidation of polyunsaturated fatty acids of membrane lipids which leads to membrane damage and cell death. LPO levels decreased during the development period related to increased antioxidant enzyme activities. We have found that LPO levels in B.viridis decreased as 8.6-fold whereas the fall in LPO levels in R.r.ridibunda was not as greater as , it was decreased 1.41-fold during the metamorphosis period. The variations in TSA levels were different from the correlation between increased antioxidant enzyme activities and decreased LPO levels . They tended to decrease after a maximum point observed at 3^{rd} and 5^{th} week of metamorphosis period of R.r.ridibunda and B.viridis. Overall, the results showed that in the war against free radicals, the predominant terrestrial amphibian B.viridis is more successful in its higher antioxidant enzyme activities than the predominantly aquatic amphibian R.r.ridibunda.

Glutathione is ubiquitous in all living cells. It has several important functions including protection against oxidative stress caused by reactive oxygen species such as hydroxyl radical and singlet oxygen.

GSH is replenished from GSSG by GSH-red using NADPH generated by enzymes such as G6DPH. These reactions are linked in concerted fashion to maintain relatively high t-GSH/GSSG ratio which ultimately protects the cells from damage from H_2O_2 and lipid hydroperoxides .

These results can be explained with over production of free radicals are higher in the predominantly terrestrial amphibian because of its evolutionary superiority. On the other hand, over production of reactive oxygen species may be one of the reasons of the most dramatic morphological changes seen in amphibians the metamorphosis; the gills of larvae disappaer, adult develop lungs etc. The most markedly changes can be observed in tail. Because of the chronological

disapperance of the constituent cells in the tail, the mechanism underlying this phenomenon has been postulated to involve "programmed cell death". In fact, the apoptotic degradation of muscle cells has been observed histologically in the tail of the tadpole during metamorphosis . According to the our results, GSH-Px activities significantly increased from 0.25 ± 0.045 to 3.45 ± 0,21 and from 0.89 ± 0.125 to 4.1 ± 0.21 IU/mg, respectively in R.r.ridibunda and B.viridis. Di Ilio et al reported that Selenium dependent and selenium independent GSH-Px activity variations of Bufo bufo fluctuated independently during its development period. LPO levels decreased with increased GSH-Px activityin both species' tadpoles. The fall as fold for LPO levels in R.r.ridibunda and B.viridis were 1.41 and 8.6, respectively. This fold differences between the species caused by higher GSH-Px activity determined in B.viridis when the compared with R.r.ridibunda. However, there is no study on LPO variations of amphibian development so it is very hard to compare with other research.

On the other hand, elevated GSH-Px activity, may support the fall for the LPO levels during the metamorphosis period for both species. The fall determined in the LPO levels for both species during the development period can be explained with elevated GSH-Px activities especially increased ROS scavenger enzymes after the 5^{th} week. Since antioxidant enzyme activities of B.viridis was higher than in R.r.ridibunda, free radical production in metabolism of B.viridis may be higher than R.r.ridibunda. Accordingly, it can be said that B.viridis is more tolerant to reactive oxygen species than R.r.ridibunda larvae.

All antioxidant enzyme activities in tissues of adult B.viridis were higher than in R.r.ridibunda. When the season were concerned, all antiocidant enzymes were higher in activie season than in hibernation season. Accordingly, lipid peroxidation levels were also lower in hibernation period than levels in active period. When the body legnth were concerned, all antioxidant enzymes activities of 5-7 cm frogs of both species were higher than in 3-5 cm (young) specimens. While maximum SOD and CAT activities were determined in liver of B.viridis in the active period as 46 ±2.3 and 2631 ± 130.1 IU/mg, respectively, maximum

GSH-Px activity was the highest in the heart of B.viridis 40.2 ± 1.5 IU/mg in the active period. When the membrane Lipid peroxidation levels were concerned, the tissues which having maximum antioxidant enzymes also had lower lipid peroxidation levels. According to results, in the war against to oxygen derived free radicals, B.viridis is more succesful than R.r.ridibunda since its higher antioxidant enzyme activities and lower lipid peroxidation levels.

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