A FREE RADICAL HYPOTHESIS OF LEAD POISONING AND INBORN PORPHYRIAS ASSOCIATED WITH 5-AMINOLEVULINIC ACID OVERLOAD

Etelvino J. H. Bechara*, Marisa H. G. Medeiros, Hugo P. Monteiro, Marcelo Hermes-Lima, Benedito Pereira, Marilene Demasi, Cristine A. Costa, Dulcineia S. P. Abdalla, Janice Onuki, Caio M. A. Wendel and Paolo Di Mascio
Instituto de Química da USP - CP 20780 - 01498-970 - SP - Brazil

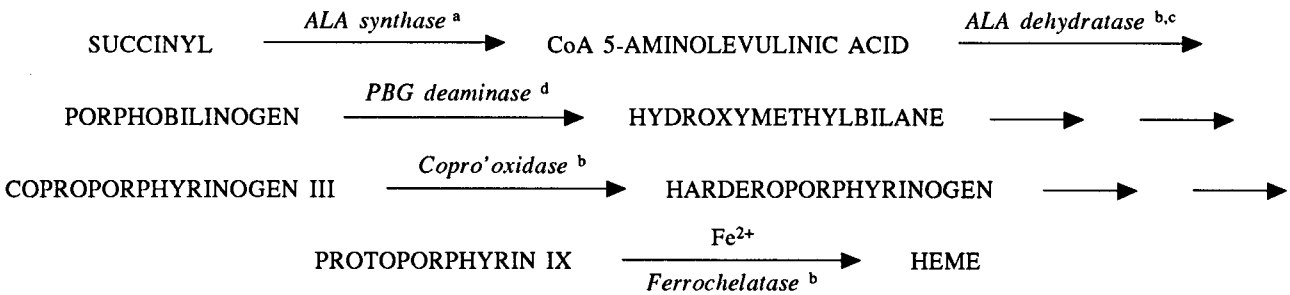
This work reviews *in vitro* and *in vivo* studies carried out in our laboratories on a role for accumulated ALA as a common potential endogenous source of deleterious oxyradicals and H_2O_2 in lead poisoning, acute intermittent porphyria (AIP) and hereditary tyrosinemia. In summary: (i) ALA undergoes transition metal-catalyzed oxidation by molecular oxygen to give $O_2^{\cdot}$, H_2O_2, $HO^{\cdot}$ radicals, NH_4^+ ions and 4,5-dioxovaleric acid; (ii) oxyHb co-oxidizes to metHb in the presence of ALA; (iii) ALA induces lipid peroxidation of cardiolipin-rich vesicles and single strand breaks in plasmid DNA; (iv) ALA causes Ca^{2+}-mediated, Mg^{2+}-antagonized rat liver mitochondrial permeabilization, (v) ALA chronically-treated rats suffer early fatigue under imposed swimming-training and increased muscle and liver glycolytic metabolism; (vi) increased chemiluminescence emission from exposed red muscle, brain and liver of ALA-treated rats signals augmented lipid peroxidation; (vii) the brain of ALA-treated rats exhibits increased levels of oxidized proteins, ferrous species and TBARS; (viii) synaptosomes and synaptic membranes of ALA-treated rats present altered ability to capture Ca^{2+} ions and to bind GABA, respectively; and, finally, (ix) AIP patients and lead-exposed workers present augmented erythrocytic levels of SOD and GSH-Px, interpreted as a protective response against ALA-generated reactive oxygen species; ALA-treated rats also showed increased activity of CuZnSOD in brain, red muscles and liver. Our unifying hypothesis for the etiology of those porphyric syndromes is that overproduced ALA may generate $O_2^{\cdot}$ and $HO^{\cdot}$ radicals *in vivo*, which act as oxidizing weapons against biomolecules (proteins, DNA, lipids) and supramolecular structures (mitochondria, endoplasmic reticulum, other membranes), thus impairing vital functions of cells (Ca^{2+} and iron homeostasis, binding of receptors, respiration) and, consequently, the functionality of several organs.

Keywords: 5-aminolevulinic acid; reactive oxygen species; lead poisoning; intermittent acute porphyria; porphyria; oxidative stress.

1. THE PORPHYRIAS

The porphyrias are defined as acquired and inborn diseases linked to enzymatic deficiencies - inhibition or insufficiency - in the heme biosynthetic pathway, resulting in excessive production and excretion of formed porphyrins and their precursors (Scheme 1)[1,2] .

These disorders are traditionally classified on the basis of (i) the main organ source of excessive porphyrin metabolites - the liver or the erythropoietic tissues; (ii) the occurrence or not of acute attacks; or (iii) whether cutaneous (photochemical), neurological or both syndromes are produced. Of particular interest to our research group are those characterized by accumulation and urinary excretion of 5-aminolevulinic



[a] Negative feedback inhibition by heme; in AIP, activated by certain drugs and metabolites.
[b] Inhibited by lead (lead poisoning).
[c] Inhibited by succinylacetone (hereditary tyrosinemia).
[d] Deficient biosynthesis in AIP.

***Scheme 1.** Heme Biosynthetic Pathway.*

acid (ALA), the first metabolite in the heme biosynthetic pathway and a potential endogenous source of reactive oxygen species (ROS) *in vivo*, as will be demonstrated here.

In mammalian cells, ALA is synthesized from glycine and succinylCoA by ALA synthase in the mitochondrial matrix and then diffuses to the cytoplasm where it initiates the biosynthesis of porphyrins[3]. This reaction is rate limiting and is under negative feedback inhibition by the heme. An alternative pathway for ALA biosynthesis, operative in plants but minor in animals, involves a transamination reaction between L-alanine and 4,5-dioxovaleric acid (DOVA), catalyzed by DOVA transaminase[4]. The biological fate of ALA in living organisms is principally the biosynthesis of chlorophylls (only in plants) and the heme-proteins and -enzymes[5]. Heme degradation products such as biliverdin and bilirubin may act as plasma anti-oxidants[6].

Among the porphyrias related to ALA overload without cutaneous lesions, we mention[2]: (i) **lead poisoning** (plumbism, saturnism), attributed to lead inhibition of ALA dehydratase, coproporphyrinogen oxidase and ferrochelatase with consequent accumulation of ALA, coproporphyrin and zinc protoporphyrin, respectively; (ii) **acute intermittent porphyria** (AIP), an inherited disorder characterized by deficient biosynthesis of porphobilinogen (PBG) deaminase leading to elevated PBG and ALA levels in blood and urine; and (iii) **hereditary tyrosinemia**, consequence of an inborn deficiency of fumarylacetoacetate hydrolase of tyrosine catabolism, resulting in increased production of succinylacetone which strongly competes with ALA (K_i = 2-3 x 10^{-7} M *vs* K_m = 2-5 x 10^{-4} M)[7] in the ALA dehydratase reaction, leading to accumulation of ALA (Scheme 1). In all cases, increased urinary excretion of ALA occurs and, in the former two cases, 10-100-fold elevated plasmatic ALA concentration were measured[8,9].

The medical literature describes many similarities in the clinical expression (**acute episodes of neuromuscular and psychiatric manifestations**) of these three types of porphyria[2,10-13], particularly between AIP and hereditary tyrosinemia[14].

AIP prevalence is globally estimated as 1-3 cases/100,000 inhabitants[15]. The main clinical features of AIP are acute attacks of abdominal pain, constipation, severe motor disturbances, tachycardia, hypertension and psychiatric manifestations, frequently hallucinations[2,3]. Residual paralysis, hypertension, and renal failure are long-term sequelae. The acute crises are precipitated by a wide variety of endogenous and xenobiotic compounds, including steroid hormones, all anticonvulsivants (eg., barbiturates), anesthetics and sulfonamides. More recently, a significantly higher incidence of hepatomes in AIP carriers who experienced several acute attacks was reported[16-18].

Lead poisoning is one of the major environmental and occupational problems nowadays. It afflicts principally children with pica habits, potters, shoe makers, a number of industrial workers and populations of urban areas with high industrial density and where lead-containing fuels are still employed[10,11-13]. Typical symptoms of lead poisoning include anemia, kidney failure, abdominal pain, peripherical paralysis and brain damage sometimes accompanied by encephalitis, altered behaviour, inattention, IQ deficits, seizures, and hallucinations[13].

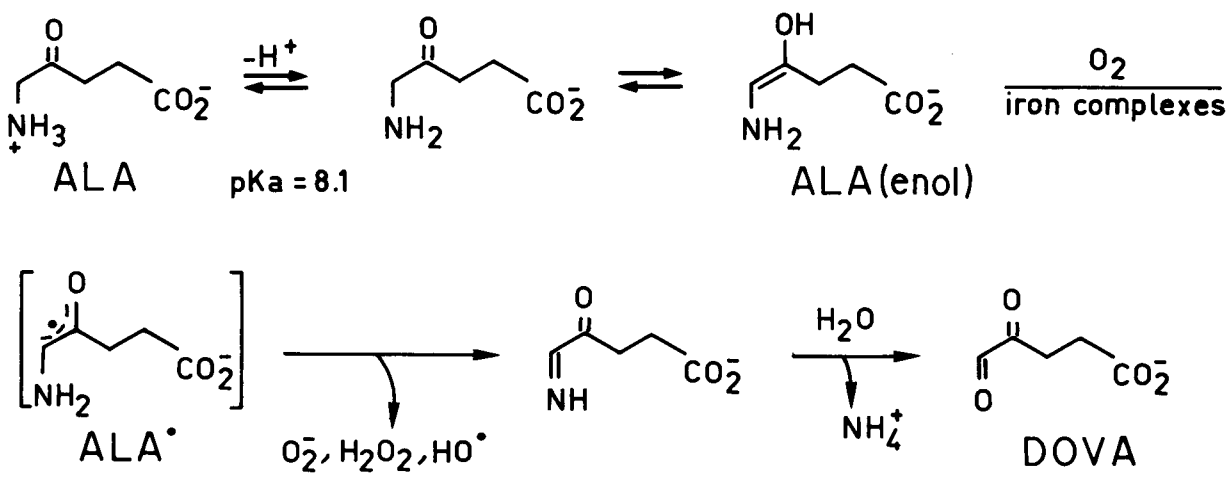
Liver and kidney damage occur in hereditary tyrosinemia carriers and the neuropsychiatric symptoms closely resemble those of AIP[3,19]. Liver cirrhosis and tumors have also been reported in patients with the chronic form of this disease.There is a high prevalence of the trait in the French-Canadian population of Quebec (8/100,000 births)[20].

This paper provides *in vitro* and *in vivo* evidence which support the hypothesis that transition metal ion - catalyzed generation of ROS ($O_2^{\cdot-}$, superoxide; H_2O_2, hydrogen peroxide; and $HO^{\cdot}$, hydroxyl radical) by overloaded ALA may be etiologic agents of the clinical symptoms of the chemical and inherited porphyrias discussed here[21], with mitochondria and synaptic membranes as their main targets.

2. 5-AMINOLEVULINIC ACID, A CHEMICAL SOURCE OF ROS

Similarly to *alpha*-hydroxy carbonyl compounds, like dihydroxyacetone[22], glyceraldehyde and glucose[23,24], ALA (an *alpha* -aminoketone) undergoes enolization[25,26] and subsequent oxidation by dissolved molecular oxygen, in slightly alkaline buffers, to yield DOVA, NH_4^+ ions, and H_2O_2 (Scheme 2)[25,27].

This reaction was shown to be efficiently catalyzed by added iron complexes like $Fe^{2+,3+}$-EDTA, $Fe^{2+,3+}$-ATP, met- and oxy-hemoglobin (Hb)[25] and ferritin (unpublished results). Non-redox active metal ions like Mg^{2+}, Ca^{2+} and Cd^{2+} at millimolar concentrations and micromolar Pb^{2+} had no effect whatsoever on the rate of oxygen uptake by ALA-containing solutions; millimolar Zn^{2+} had some inhibitory effect (unpublished results). Spectrophotometric studies in the visible absorption region of Hb showed complete co-oxidation of oxyHb (6 μM) to metHb in the presence of 6 mM ALA within 30 min (optimum pH *ca.* 8), followed by formation of hemochromes and loss of secondary structure due to the oxidation



Scheme 2. Metal-catalyzed ALA Oxidation.

of amino acid residues, indicated by CD measurements at 224 nm[27]. The involvement of $O_2^{\cdot}$, H_2O_2 and metal-catalyzed Haber-Weiss produced HO· radicals in the co-oxidation of ALA and oxyHb was evidenced by the observed inhibitory effects of added bovine superoxide dismutase (CuZnSOD), catalase and mannitol, respectively, on both oxygen uptake and oxyHb decay curves.

$$H_2O_2 + O_2^{\cdot} \xrightarrow{Fe^{3+},\ Cu^{2+}} HO^{-} + HO\cdot + O_2 \quad \text{(Haber-Weiss reaction)}$$

The intermediacy of superoxide species in ALA autoxidation was further demonstrated by parallel reduction of added ferricytochrome c, which was inhibited in the presence of CuZnSOD. Semicarbazide, known to react with superoxide, also blocks the metal-catalyzed ALA oxidation (unpublished results). Direct evidence for the generation of oxy- and carbon-centered radicals during the aerobic oxidation of ALA in both the presence and absence of oxyHb was provided by spin-trapping experiments (Fig. 1)[25], done in collaboration with Dr. O. Augusto (Universidade de São Paulo) .

Hydroxyl radical formation by ALA oxidation was attested to by the observed characteristic 1:2:2:1 four line ESR spectrum of the DMPO-hydroxyl adduct ($a_N = a_H = 14.87$ G) (Figure 1) and the six-line spectrum of DMPO-methyl adduct ($a_N = 16.56$ G; $a_H = 23.68$ G) upon addition of DMSO to the reaction mixture (methyl radicals arise from reaction of HO· with DMSO)[25]. Addition of either CuZnSOD or catalase quenches the ESR signal, showing again that HO· radicals are formed from the Haber-Weiss reaction of ALA-generated $O_2^{\cdot}$ and H_2O_2. On the other hand, incubation of ALA in the presence of the spin trap POBN gave rise to a stable ESR signal with $a_N = 15.52$ G and $a_H = 2.96$ G, which can be assigned to a POBN-carbon centered adduct[25]. This is also quenched by addition of CuZnSOD and thus consistent with the intermediacy of an enoyl ALA radical ($ALA^{\cdot}$ in Scheme 2) in the propagation of ALA oxidation.

3. ALA-PROMOTED OXIDATION OF LIPOSOMES AND DNA

Knowing that ALA produces HO· by aerobic oxidation, we decided to test its ability to initiate lipid peroxidation. In collaboration with Dr. P. Oteiza (Universidad de Buenos Aires), we demonstrated that 0.1-3 mM ALA promoted, albeit slowly (time scale of a couple of hours), the lipid peroxidation of

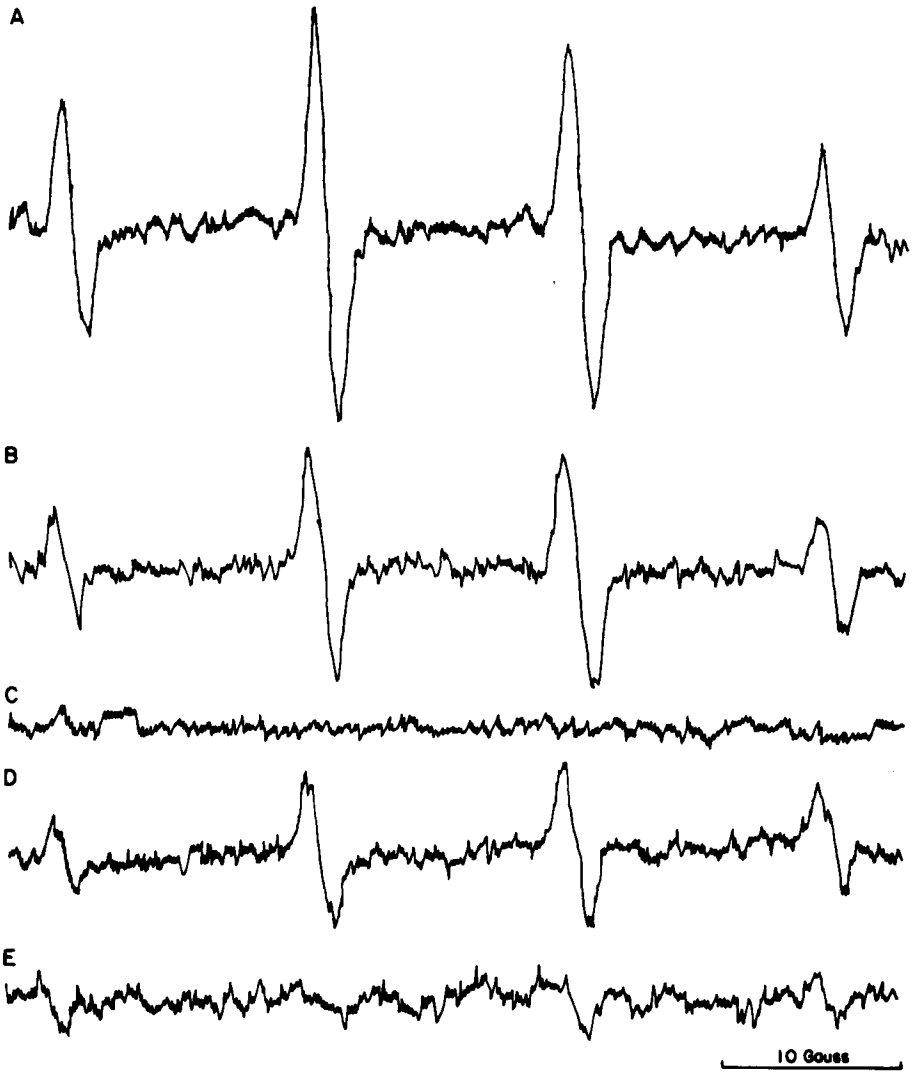


Figure 1. *ESR spectra of DMPO-radical adducts obtained after 30 min incubation of ALA (6 mM) and DMPO (150 mM) in 0.1 mM DTPA-containing 0.10 M phosphate buffer, pH 7.8, at 36°C, both in the absence and presence of oxyHb (14 μM): (A) ALA plus oxyHb; (B) ALA alone; (C) ALA/oxyHb plus catalase (2.0 μM); and ALA/oxyHb plus SOD (10 μg/ml, D and 100 μg/ml, E).*

phosphatidylcholine:cardiolipin (80:20) liposomes as evaluated by the formation of conjugated dienes and thiobarbituric reactive substances (TBARS) and by the release of encapsulated carboxyfluorescein[28]. *Alpha*-tocopherol, an efficient oxyradical scavenger, inhibited the TBARS formation and prevented fluorescer release, ratifying that HO· radicals are indeed formed during ALA oxidation and promote the liposome permeabilization. Cardiolipin-rich vesicles were used to mimic the inner mitochondrial membrane, known to be particularly rich in cardiolipin[29]; rat liver mitochondria were shown to be injured by added ALA (discussed in the next section). Recently, Wratten et al.[30] showed that oxidized cardiolipin has a more disturbing effect on bilayer organization than oxidized phosphatidylcholine, thereby increasing microheterogeneity and facilitating the lipid peroxidation propagation. Also noteworth of mention in this regard are findings that the position of the *alpha*-benzoylacetanilide keto-enol equilibrium in the presence of liposomes favors the enol form in the lipid phase[31]. If this also occurred with ALA in the interface of synthetic and biological membranes, the higher concentration of both O_2 and ALA in the lipid phase should make the membranes more prone to lipid peroxidation.

Motivated by literature reports on the higher incidence of primary liver carcinoma in AIP patients who underwent a series of acute crisis than in asymptomatic carriers[16-18], we also decided to challenge isolated DNA with ALA. Preliminary results obtained in collaboration with Dr. P. Di Mascio (Universidade de São Paulo) clearly show that exposure of supercoiled plasmid pBR322 DNA to 0.01-3 mM ALA led to DNA single strand breaks, revealed by formation of the corresponding plasmid open circular form. That HO· radicals produced by the Haber-Weiss reaction of ROS generated by iron-catalyzed ALA oxidation were responsible for DNA single strand breaks was evidenced by the observed inhibitory effect of catalase, CuZnSOD, mannitol or DTPA added to the plasmid/ALA system (Bechara and coworkers, in preparation for publication). These results may be relevant with regard to mitochondrial DNA mutations that may contribute to cellular aging and promotion of cancer[32].

4. *IN VITRO* STUDIES WITH RAT LIVER MITOCHONDRIA

In both lead poisoning and AIP disorders, extensive liver and kidney mitochondrial damage, respectively, were observed[10,33]. This led us, in collaboration with Dr. A.E. Vercesi (Universidade Estadual de Campinas, São Paulo State), to study possible deleterious effects of ALA-generated ROS upon rat liver mitochondria and the corresponding mechanisms of oxidative insult.

Indeed, addition of millimolar ALA was shown to disrupt the mitochondrial transmembrane potential, to promote Ca^{2+} release from the intramitochondrial matrix, to release the state-4 respiration and consequently to cause mitochondrial swelling[34]. The mitochondrial permeabilization was abolished by catalase, CuZnSOD, and *ortho*-phenanthroline, an iron chelator, which is in agreement with the idea that the Haber-Weiss reaction of ALA-generated $O_2^{\cdot-}$ and H_2O_2 drives the mitochondrial injury; accordingly, H_2O_2 alone induces damage poorly.

Furthermore, we found that mitochondrial swelling occurs at ALA concentrations as low as 50-100 μM, the level assumed to occur in the liver of AIP patients (about 60 μM) on basis of the following findings: (i) in two cases of AIP with overwhelming neuropathy, the maximum plasma ALA concentration was found to be 9 and 12 μM, which is approx. 100-times above the mean normal value[35] and (ii) when studying ALA i.p. injected rats (40 mg/kg body weight), McGillion et al.[36] found that ALA distributes among several organs, including brain and liver, where the ALA concentration is about 6-times higher than in the plasma. The actual concentration of ALA in the liver of AIP patients is not known. Plasma ALA concentrations were measured in individuals exposed to lead and compared to a control group: 3.5 and 14 μM ALA was found for individuals with <30 and >30 μg Pb/dl blood, respectively (control, 3.5 μM ALA)[8]. This means, taking into consideration the data for hepatic ALA of treated rats mentioned above[36], that lead poisoned subjects can have hepatic ALA levels as high as 80 μM, a concentration shown to cause oxidative injury to mitochondrial preparations.

Removal of Ca^{2+} (10 μM) from the suspension of isolated rat liver mitochondria by adding EGTA abolished both the ALA-induced transmembrane potential collapse and the mitochondrial swelling[37]. Upon addition of ruthenium red (an inhibitor of Ca^{2+} influx) prior to mitochondrial energetization with succinate, the ALA-induced mitochondrial swelling was prevented, demonstrating the deleterious role of intra-matrix Ca^{2+} in the oxidative damage. The mediation of Ca^{2+} in the inner mitochondrial membrane permeabilization promoted by several pro-oxidants, such as diamide and *t*-butyl hydroperoxide, is reviewed by Dr. A.E. Vercesi in this issue of *Quimica Nova*. That the ALA-induced, Ca^{2+}-mediated increase of mitochondrial permeability could be caused by the loss of membrane-bound Mg^{2+}, known to be important for membrane integrity[38], was indicated by the antagonistic protective effect of 0.8-5.0 mM Mg^{2+} added to the ALA-damaged mitochondria (intracellular free Mg^{2+} is *ca.* 1 mM).

Under our experimental conditions, complete oxidative damage to mitochondria by added ALA was generally attained within 10-15 min. If either catalase, *ortho*-phenanthroline, EGTA or dithiothreitol (DTT, a thiolic reducing agent) is added to the ALA- and mitochondria-containing medium at the initial stage of the oxidative damage, one can observe almost complete recovery of the transmembrane potential[39]. If the reagent addition is done after 8 min, when the potential has almost completely collapsed, then only EGTA can promote complete recovery. Gel electrophoretic analyses of the ALA/mitocondria system after 20 min incubation revealed extensive formation of thiol-protein aggregates (disulfide cross-linked proteins), which disappear upon pre-treatment with DTT. Taken together, these data indicate that ALA-generated ROS cause calcium-mediated mitochondrial permeabilization *via* the oxidation of thiol-proteins in an early stage. Lipid peroxidation, demonstrated to be a relatively slow process with cardiolipin-rich vesicles (time scale of hours), may follow this process, making the oxidative lesion irreversible .

The idea that liver mitochondria could be a potential target for ROS generated by iron-catalyzed ALA aerobic oxidation is also supported by cytochemical and ultrastructural studies of liver in AIP and porphyria cutanea tarda carried out by Biempica et al.[33]. When analyzing liver biopsy samples of four AIP patients, these authors found numerous lipid droplets, abundant intramitochondrial and cytoplasmic ferritin granules, large lipofuccin bodies, odd-shaped mitochondria (from bizarre to ghost forms), hyperplasia of smooth endoplasmic reticulum and autophagic vacuoles containing well-preserved mitochondria. It is tempting to attribute these abnormalities to oxidative damage triggered by over-produced ALA and accumulated (Fe^{3+})ferritin in the hepatocytes. Potentially harmful concentrations of ferrous chelates (such as Fe^{2+}-ATP and Fe^{2+}-citrate) could be formed from (Fe^{3+})ferritin and ALA-generated $O_2^{\cdot-}$. Recently, Minotti et al.[40] raised the possibility that ALA-generated $O_2^{\cdot-}$ may be important in the mobilization of iron embedded in the endoplasmic reticulum towards the mitochondrion, where incorporation of iron into the porphyrin ring is operated by ferrochelatase.

In collaboration with Dr. H. Sies and Dr. S. Soboll (both from Düsseldorf University), we have also examined the possibility that the product of ALA oxidation, 4,5-dioxovaleric

acid (DOVA), might be metabolized to succinylCoA *via* the Krebs cycle and then be used up for ALA re-synthesis. If this were actually true, then the coupled operation of ALA oxygen-dependent oxidation and the Krebs cycle would greatly increase the intramitochondrial production of ROS. Using [5-^{14}C]ALA and rat liver mitochondrial preparations and following ALA catabolism by measuring $^{14}CO_2$, we demonstrated that external ALA is taken up by the mitochondria but, instead of undergoing the chemical ROS-generating reaction, it is deaminated by monoaminooxidase and transaminase activities before undergoing to a very small extent (0.2%), decarboxylation by the Krebs cycle[41]. Thus, intramatrix ALA probably does not cause oxidative damage and its fate is to leave the mitochondria to be used for heme biosynthesis. One should also keep in mind that the inner space of mitochondria is well protected against ROS by chemical and enzymatic anti-oxidants, such as reduced glutathione (GSH), ubiquinone, vitamin E, MnSOD and glutathione -peroxidase (GSH-Px), which could also break the ALA oxidation chain.

5. *IN VIVO* STUDIES WITH ALA-TREATED RATS

McGillion et al.[36] demonstrated that ALA injected intraperitonially in rats (40 mg/kg body weight) distributes among and accumulates in several organs (20-fold in brain, 10^2-fold in kidney and 10^3-fold in liver) three hours after injection and slowly decays to the normal level within 3-8 days; liver ALA concentration is about 6-fold higher than in blood. We thus thought that chronically ALA-treated rats (40 mg/kg body weight every two days during two weeks to mantain continuously elevated ALA in the plasma) should be an adequate model to represent the pathological states of AIP carriers under acute crisis and lead poisoned individuals with >30 μg Pb/100 g blood, where the blood ALA level increases up to 10^2-fold[8,9].

After chronic ALA treatment of rats, we found: (i) elevated serum lactate (141%) and free fatty acid (33%) levels, (ii) increased mobilization of glycogen from the liver (50%), the soleus (red, mitochondria rich) muscle (83%) and the red portion of gastrocnemius muscle (43%), (iii) no changes in the white (mitochondria poor) portion of the gastrocnemius muscle, (iv) loss of mitochondrial enzymatic activities such as soleus muscle citrate synthase activity (45% in swimming-trained rats and 180% in sedentary rats) and MnSOD (250% in liver, 78% in soleus muscle, and 51% in the red portion of gastrocnemius muscle)[42]. An endurance exercise training program coupled to the ALA-treatment was used to intensify the enzymatic diferences triggered by ALA, as mitochondrial proliferation is known to be induced by exercise-training. This work was carried out in collaboration with Dr. R. Curi (Universidade de São Paulo) and Dr. E. Kokubun (Universidade Estadual Paulista, Rio Claro).

Together these data testify in favor of increased glycolytic, non-aerobic metabolism due to the impairment of mitochondrial functions by the pro-oxidative action of ALA. Accordingly, ALA-treated rats reached fatigue under swimming training significantly earlier than the control, non-treated ALA group (40 *vs* . 90 min). With regard to the cytosolic CuZnSOD, we found increased activity in brain (61%), liver (148%) and the red fibers of the gastrocnemius muscle (57%), and decreased activity in soleus (-184%). The observed increase in activity of CuZnSOD was interpreted as a protective response against the deleterious effects of ALA.

Preliminary chemiluminescence experiments carried out *in vivo* [43] in collaboration with Dr. S. Llesuy (Universidad de Buenos Aires) provided data that parallel those mentioned above. Treatment of rats with two doses of 40 mg ALA/kg body weight (four days) rendered increased levels of chemiluminescence intensity in liver (4-fold), red muscle (5-fold) and brain (5-fold), indicative of increased peroxidative activity (unpublished results) and thus ALA-driven oxidative stress.

More recently, we initiated collaboration work with Dr. R. De Luccia (Universidade de São Paulo) and Dr. P. Oteiza (Universidad de Buenos Aires) with the aim of characterizing the ALA pro-oxidant effect in brain tissue, which could be related to the neuropsychiatric syndromes associated with lead poisoning, AIP and tyrosinosis. We decided to compare several biochemical parameters associated with oxidative stress in the brain of ALA-treated and non-treated rats, such as: Ca^{2+} uptake by cortical synaptosomes; antioxidant enzyme (SOD and GSH-Px) activities, TBARS and both carbonyl and disulfide protein formation in total brain homogenate; iron mobilization to the brain; and ^{3}H-muscimol (a gabaergic receptor ligant) binding capacity of synaptic membranes prepared from whole brain. Under acute treatment (just one dose of 40 mg ALA/kg body weight), an increased ratio of ferrous/total iron species was observed in the brain homogenate. After prolonged treatment (one dose on alternate days during two weeks), all other parameters were found to be significantly altered, including the total content of iron species and the ratio ferrous/total iron (in preparation for publication). These results again argue in favor of ALA-triggered oxidative stress in brain. It is thus tempting to propose that the neuropsychiatric manifestations of the acquired and inherited porphyrias studied here are linked to both the well-demonstrated competitive binding of ALA to gabaergic receptors and competition with GABA uptake by nerve cells[44,45] and, subsequently, to oxidative nerve damage. This could perhaps explain the severe nerve damage and demyelinization reported to occur in AIP and lead poisoning. Interesting in this regard are Tishler et al.'s findings[46] that some AIP carriers could develop **chronic** psychiatric manifestations common to other psychiatric pathologies, without **acute** episodes, suggesting continous oxidative damage to the brain.

6. STUDIES WITH AIP PATIENTS AND LEAD-EXPOSED WORKERS

Since the late seventies, several reports of abnormally high levels of anti-oxidant enzymes, particularly SOD, in the red blood cells of individuals with mental aberrations (Down's syndrome[47], autism[48], schizophrenia and manic-depression[49] and several other psychiatric disorders[50]) were suggested to be indicative of an oxidative disbalance in the central nervous system. At that time, knowing that AIP carriers present a typical neuropathy and undergo episodes of hallucinations during acute attacks and that lead poisoning symptoms include behavioural alterations and mental deficiencies, we extended these studies to the porphyric diseases.

In collaboration with P. Marchiori (Hospital das Clínicas, São Paulo), we examined latent and symptomatic AIP carriers with regard to the blood levels of CuZnSOD, GSH-Px and catalase. Significantly higher levels of SOD (32 and 100% in latent and symptomatic patients, respectively) and of GSH-Px (about two-fold in both cases) were found as compared to a control group (Table 1)[51]. Treatment of one AIP patient with intravenous hematin for four consecutive days did not ameliorate the neuropathy or affect the erythrocytic level of SOD. An evaluation of erythrocytic SOD and GSH-Px in non-exposed (<15 μg Pb/100 g blood; <120 μg protoporphyrin-IX/100 ml erythrocytes) and exposed workers (15-150 μg Pb/100 g blood; >120 μg PP-IX/100 ml erythrocytes) of several industries of metropolitan São Paulo showed significantly augmented activities of both enzymes: 85% and 140% for SOD and GSH-Px, respectively (Table 2)[52]. The dose-response relationship between erythrocytic SOD and lead concentrations parallels that obtained for urinary ALA *vs.* blood lead[10]. The highest SOD value (1960 U/g Hb) was found in a janitor

Table 1. Erythrocyte SOD, GSH-Px and Catalase Values in AIP Patients.

Enzymes	Group		
	Control	IAP, asymptomatic	IAP, acute crisis
SOD (U/g Hb)	490 ± 86 (32)	650 ± 41 (8)	992 ± 95 (8)
GSH-Px (μmol NADPH/min/g Hb)	10.6 ± 2.7 (20)	20.0 ± 2.5 (4)	18.5 ± 0.9 (3)
Catalase (μg/g Hb)	2020 ± 650 (30)	2500 ± 170 (6)	1638 ± 560 (4)

Results are presented as $\bar{X}$ ± SD (n).

Table 2. Erythrocyte SOD and GSH-Px Levels in Lead-exposed Individuals.

Groups	SOD (U/g Hb)		GSH-Px (μmol NADPH/min/g Hb)	
	n	$\bar{X}$ ± SD	n	$\bar{X}$ ± SD
Factory A				
Exposed	12	996 ± 382 [a]	11	31.0 ± 19.4 [a]
Non-exposed	26	612 ± 139	21	9.3 ± 3.2
Factory B				
Exposed	24	1176 ± 365 [a]	28	34.2 ± 14.5 [b]
Non-exposed	21	575 ± 98	29	20.7 ± 9.2
Various Industries				
Exposed	26	861 ± 288 [a]	27	53.5 ± 34.0 [a]
Control	27	467 ± 94	19	23.9 ± 11.2

[a] $p < 0.01$; [b] $p < 0.05$

(three-year exposure time) who was responsible for sweeping lead-containing dust from the prodution site of an electric cable manufacturing plant. This work involved the participation of Dr. A.S.A. Arcuri (Division of Industrial Hygiene and Safety, Serviço Social da Indústria - SESI, São Paulo). This survey of AIP patients and lead-exposed workers with regard to the anti-oxidant enzymes was actually the starting point for the work described in this review and provided arguments to support our free radical hypothesis of chemical and inborn porphyrias characterized by elevated plasmatic ALA.

7. IS ALA THE COMMON ETIOLOGIC AGENT IN AIP, HEREDITARY TYROSINEMIA AND LEAD POISONING?

On a biochemical basis, the relationships between the clinical symptoms of lead poisoning, AIP and tyrosinemia are yet to be established. The only common factor in these diseases is the elevation of plasma ALA due to the inhibition or hypoproduction of certain enzymes of the heme biosynthetic pathway. At the subcellular level, they exhibit liver or kidney mitochondrial damage and, in the case of AIP, demyelinization of nerve cells. Kidney and liver insufficiency, hepatomas and neuropsychiatric manifestations are consequences common to those three types of porphyria .

The neurological manifestations of AIP have long been attributed to the competition between the excess ALA and *gamma*-butyric acid (GABA) for binding sites of synaptic membranes[44,45]. In the case of lead poisoning, the molecular bases of the syndrome are more complex and multifactorial, since Pb^{2+} ions inhibit the activity of a large number of thiol-proteins and -enzymes such as those of the heme biosynthetic pathway shown in Scheme 1, hemoglobin[53], (Ca^{2+},Mg^{2+})[54] and (Na^{+},K^{+})[55] ATPases, adenyl cyclase[56], glucose-6-phosphate dehydrogenase and phosphofructokinase[57]. The possibility that Pb^{2+} ions can directly promote oxidative damage in several biological systems has also been demonstrated; e.g., Pb^{2+}-catalyzed oxidation of oxyHb to met-Hb and ROS[53] and Pb^{2+}-stimulated, iron-initiated lipid peroxidation of phospholipid liposomes, erythrocytes and microsomes[58]. With regard to pro-oxidant properties of ALA, the literature also describes ALA-induced photo-oxidative damage to animal and vegetal tissues, possibly *via* oxyradicals and singlet oxygen produced from electronic excitation of the porphyrins biosynthesized from ALA. Herbicidal[59], insecticidal[60] and tumor killing[61] properties of ALA under illumination have also been described.

Recently, ALA was found to cause transient (5 min) and dose-dependent relaxation of rat aortic rings, suggesting that it interferes in the mechanism of NO-mediated modulation of the vascular tone[62]. At first sight, however, based on the property of ALA to generate $O_2^{\cdot-}$ species which are known to decrease the lifetime of NO - the endothelium-derived relaxing factor (EDRF)[63]- one would predict an opposite effect of ALA on the aortic rings.

In this paper we present published and in progress research information from our laboratories, with *in vitro* and *in vivo* systems, which strongly argues for a role of ALA as a potential endogenous source of deleterious oxyradicals and H_2O_2

when accumulated in the porphyric states considered here. In summary: (i) ALA undergoes transition metal-catalyzed oxidation by molecular oxygen, propagated by $O_2^{\cdot}$ and ALA enoyl species, to give H_2O_2, $HO^{\cdot}$ radicals, NH_4^+ ions and DOVA; (ii) oxyHb co-oxidizes to metHb in the presence of ALA; (iii) ALA induces lipid peroxidation of cardiolipin-rich vesicles and single strand breaks in plasmid DNA; (iv) ALA causes Ca^{2+}-mediated and Mg^{2+}-antagonized rat liver mitochondrial permeabilization, revealed by the release of State-4 respiration, collapse of the transmembrane potential and, consequently, swelling, probably *via* cross-linking of membrane thiol-proteins; (v) chronically ALA-treated rats suffer early fatigue under imposed swimming-training and increased muscle and liver glycolytic metabolism, as indicated by various biochemical parameters; (vi) increased lipid peroxidation in the red muscle, brain and liver of ALA-treated rats is indicated by increased chemiluminescence emission from the exposed organs; (vii) the brain of ALA-treated rats exhibits elevated contents of oxidized proteins, iron and TBARS; (viii) synaptosomes and synaptic membranes of ALA-treated rats present altered ability to capture Ca^{2+} ions and to bind GABA, respectively; and, finally, (ix) AIP patients and lead-exposed workers present augmented erythrocytic levels of SOD and GSH-Px which may indicate an adaptive, protective response against elevated ROS-generated ALA; ALA-treated rats also showed increased activity of CuZnSOD, measured in brain, red muscles and liver.

If ALA, synthesized in the mitochondria and distributed and accumulated in several organs, indeed represents a common dangerous source of ROS in AIP, lead poisoning and tyrosinemia, this role requires that ALA be enolized first to the oxidizable form. That is, enolic ALA could not be stored or circulated among the organs without causing damage. Electrophoresis of [5-^{14}C]ALA-incubated human serum did not indicate any association of ALA, possibly in the keto, non-oxidizable form, with plasmatic proteins (unpublished results). In any case, however, plasma anti-oxidants (vitamin E, ascorbate, urate, bilirubin, carotenoids, glucose and proteins like albumin, caeruloplasmin, transferrin, haptoglobin, etc.)[64,65] are expected to block the propagation of ALA oxidation, if any, and provide protection against ROS formed.

In **conclusion**, based on the *in vitro* and *in vivo* data reported here and considering the histopathological information in the literature, we propose an unifying hypothesis that overproduced ALA in acquired and inherited porphyrias may generate $O_2^{\cdot}$ and $HO^{\cdot}$ radicals *in vivo*, which act as oxidizing weapons against biomolecules (thiol-proteins, DNA, lipids) and supramolecular structures (mitochondria, endoplasmic reticulum, other membranes), thus compromising vital functions of cells (Ca^{2+} and iron homeostasis, binding of receptors, respiration) and, consequently, the functionality of several organs. If ALA can actually be considered as an endogenous source of $O_2^{\cdot}$ species, one might also propose that ALA may release iron from ferritin and thus provide "free iron" to catalyze oxidative damage in other cell structures[65]. Finally, the data presented here establish i.p. ALA-treated rats as a very useful model for *in vivo* studies of oxidative stress, as ALA is a normal metabolite present in all tissues and is promptly distributed among several organs.

ACKNOWLEDGEMENTS

This work has been supported by grants and fellowships from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT). The co-authors of this review include former and present graduate (M.H.G.M., H.P.M., M.H.L., B.P., M.D., C.A.C., D.S.P.A.) and under-graduate (J.O., C.M.A.W.) students and a research associate (P.D.M.) from our laboratories. We also thank the participation and interest of Valderez G.R. Valle (graduate student) and of Roger F. Castilho and André R. Meinicke (undergraduate-students) from Dr. A.E. Vercesi's laboratories (IB-UNICAMP). We also want to express our gratitude to many colleagues who collaborated decisively for the concretization of our ideas on the pro-oxidant role of ALA-generated ROS in porphyrias: Dr. Paulo E. Marchiori (FM-USP), Dr. Arline S.A. Arcuri (FUNDACENTRO, SP), Drs. Ohara Augusto and Adelaide Faljoni-Alário (IQ-USP), Drs. Rui Curi and R. De Lucia (ICB-USP), Dr. Eduardo Kokubun (IB-UNESP), Dr. Anibal E. Vercesi (IB-UNICAMP), Drs. Patricia Oteiza and Susana Llesuy (FFB-Universidad de Buenos Aires) and Drs. Helmut Sies and Sybille Soboll (IPC I-Universität Düsseldorf). Most of our manuscripts, including this review, have been kindly read by Dr. Frank Quina (IQ-USP). Special thanks to Adriana P. Pinto and Lydia F. Butturini for technical and secretarial assistance, respectively. During all these years, Cilento has been a dearest friend, enthusiastic scientific partner, a source of encouragement and a model of the highest ethic values.

REFERENCES

1. Brodie, M.J.; Moore, M.R. & Goldberg, A.; *Lancet* (1977), **ii**, 699.
2. Hindmarsh, J.T.; *Clin. Chem.* (1986), **32**, 1255.
3. Kappas, A.; Sassa, S. & Anderson, K.E.; **In** *The Metabolic Basis of Inherited Diseases* (Stanbury, J.B. et al., eds), McGraw-Hill, New York, p. 130 (1983).
4. McKinney, C.E. & Ades, I.Z.; *Int. J. Biochem.* (1990), **22**, 347.
5. Granick, S. & Beale, S.I.; *Adv. Enzymol. Relat. Areas Mol. Biol.* (1978), **46**, 33.
6. Stocker, R.; Glazer, A.N. & Ames, B.; *Proc. Natl. Acad. Sci. USA* (1987), **84**, 5918.
7. Sassa, S. & Kappas, A.; *J. Clin. Invest.* (1983), **71**, 625.
8. Minder, E.I.; *Clin. Chim. Acta* (1986), **161**, 11.
9. Gorchein, A. & Weber, R.; *Clin. Sci.* (1984), **72**, 103.
10. Chisolm Jr., J.J. ; *Sci. Amer.* (1971), **224**, 15.
11. Alessio, L. & Foa, V.; *Lead. Human Biological Monitoring*. Industrial Chemical Series, (Alessio, L.; Berlin, A; Roy, R. & Boni, M., eds), CECA, Luxemburg, (1983),107 p..
12. Carvalho, F.M.; Barreto, M.L.; Silvany-Neto, A.M.; Waldron, H.A. & Tavares, T.M.; *Sci. Total Environm.* (1984), **35**, 71.
13. Fergusson, J.E. ; *The Heavy Elements: Chemistry, Environmental Impact and Health Effects* (1990), Pergamon Press, Oxford, 614 p..
14. Lindblat, B.; Lindstedt, S. & Stein, G.; *Proc. Natl. Acad. Sci. USA* (1977), **74**, 4641.
15. Sack, G.H.; *J. Am. Med. Ass.* (1990), **264**, 1290.
16. Karppinen, R. & Mustajoki, P.; *Br. J. Cancer* (1988), **57**, 117.
17. Gubler, J.G.; Bargetzi, M.J. & Meyer, U.A.; *Am. J. Med.* (1990), **89**, 540.
18. Thunnissen, P.L., Meyer, J. & de Koning, R.W. ; *Neth. J. Med.* (1991), **38**, 171.
19. Strife, C.F.; Zuroweste, E.L.; Emmett, E.A.; Finelli, V.N.; Petering, H.G. & Berry, H.K. ; *J. Pediat.* (1977), **90**, 400.
20. Bergeron, P.; Laberge, C. & Grenier, A.; *Clin. Genet.* (1974), **5**, 157.
21. Hermes-Lima, M.; Pereira, B. & Bechara, E.J.H.; *Xenobiotica* (1991), **21**, 1085.
22. Mashino, T. & Fridovich, I.; *Arch. Biochem. Biophys.* (1987), **254**, 547.
23. Thornalley, P.J.; Wolff, S.P.; Crabbe, J.C & Stern, A.;

Biochem. J. (1984), **217**, 615.
24. Thornalley, P.J.; *Environm. Health Persp.* (1985), **64**, 297.
25. Monteiro, H.P.; Abdalla, D.S.P.; Augusto, O. & Bechara, E.J.H.; *Arch. Biochem. Biophys.* (1989), **271**, 206.
26. Jaffe, E.K. & Rajagopalan, J.S.; *Bioorg. Chem.* (1990), **18**, 381.
27. Monteiro, H.P.; Abdalla, D.S.P.; Faljoni-Alário, A. & Bechara, E.J.H.; *Biochim. Biophys. Acta* (1986), **881**, 100.
28. Oteiza, P. & Bechara, E.J.H., *Arch. Biochem. Biophys.*, in press.
29. Hoch, F.L.; *Biochim. Biophys. Acta* (1992), **1113**, 71.
30. Wratten, M.L.; Muscatello, U.; Pasquali-Roncheti, I.; Tomasi, A. & Vannini, V; *Free Rad. Res. Commun.* (1992), **16** (Supp.1), Abs. 12.10.
31. Ueno, M.; Katoh, S.; Kobayashi, S.; Tomoyama, E.; Ohsawa, S.; Koyama, N. & Morita, Y.; *J. Colloid Interface Sci.* (1990), **134**, 589.
32. Bandy, B. & Davison, A.J.; *Free Rad. Biol. Med.* (1990), **8**, 523.
33. Biempica, L.; Kosower, N.; Ma, M.H. & Goldfisher, S.; *Arch. Pathol.* (1974), **98**, 336.
34. Hermes-Lima, M.; Valle, V.G.R.; Vercesi, A.E. & Bechara, E.J.H.; *Biochim. Biophys. Acta* (1991), **1056**, 57.
35. Gorchein, A. & Webber, R.; *Clin. Sci.* (1984), **72**, 103.
36. McGillion, F.B.; Thompson, G.G. & Goldberg; *Biochem. Pharmacol.* (1975), **24**, 299.
37. Hermes-Lima, M.; Castilho, R.F.; Valle, V.G.R.; Bechara, E.J.H. & Vercesi, A.E.; *Biochim. Biophys. Acta* (1992), **1180**, 201.
38. Antonio, R.V., Pereira da Silva, L. & Vercesi, A.E.; *Biochim. Biophys. Acta* (1991), **1056**, 250.
39. Bechara, E.J.H.; Hermes-Lima,M.; Castillo, R.F.; Valle, V.G.R. & Vercesi, A.E.; *Free Rad. Res. Commun.* (1992); **16** (Suppl. 1), abst. 18.13.
40. Minotti, G.; *Arch. Biochem. Biophys.* (1992), **297**, 189.
41. Bechara, E.J.H.; Monteiro, H.P.; Hermes-Lima; Pereira, B.; Mrdeiros, M.H.G.; Di Mascio, P.; Curi, R.; Soboll, S. & Vercesi, A.E.; IV European ISSX Meeting, abst. 62, Bologna, Italy (1992).
42. Pereira, B.; Curi, R.; Kokubun, E. & Bechara, E.J.H.; *J. Appl. Physiol.* (1992), **72**, 226.
43. Boveris, A.; Cadenas, E.; Reiter, R.; Filipkowski, M.; Nakase, Y. & Chance, B.; *Proc. Natl. Acad. Sci. USA* (1979), **77**, 347.
44. Brennan, M.J.W. & Cantrill, R.C.; *Nature* (1979), **280**, 514.
45. Brennan, M.J.W.; Cantrill, R.C. & Kramer, S.; *Int. J. Biochem.* (1980), **12**, 833.
46. Tishler, P.V.; Woodward, B.; O'Connor, J.; Holdnook, D.A.; Seidman, L.J.; Hallett, M. & Knighton, D.; *Am. J. Psych.* (1985), **142**, 1430.
47. Agar, N.S. & Hingston, J.; *Med. J. Aust.* (1980), **1**, 556.
48. Glose, B.; Debray-Ritzen, P.; Puget, K. & Michelson, A.M.; *Nouv. Presse Med.* (1977), **6**, 2449.
49. Abdalla, D.S.P.; Monteiro, H.P; Oliveira, J.A.C. & Bechara, E.J.H.; *Clin. Chem.* (1986); **32**, 805.
50. Michelson, A.M.; Puget, K.; Durosay, P. and Bouneau, J.C.; **In** *Superoxide and Superoxide Dismutases* (Michelson, A.M., McCord, J.M. and Fridovich, I., eds), Academic Press, New York, p. 467, 1977.
51. Medeiros, M.H.G.; Marchiori, P.E. & Bechara,E.J.H.; *Clin. Chem.* (1982), **28**, 242.
52. Monteiro, H.P., Abdalla, D.S.P.; Arcuri, A.S. & Bechara, E.J.H.; *Clin. Chem.* (1985), **31**, 1673.
53. Ribarov, S.R. & Bochev, P.G.; *Arch. Biochem. Biophys.* (1982), **213**, 288.
54. Viarengo, A. & Nicotera, P.; *Comp. Biochem. Physiol.* (1991), **100**, 81.
55. Rajanna, B.; Chetty, C.S.; Stewart, T.C. & Rajanna, S.; *Biomed. Environm. Sci.* (1991), **4**, 441.
56. Nathanson, J.A. & Bloom, F.E.; *Nature* (1975), **255**, 419.
57. Lachant, N.A.; Tomoda, A. & Tanaka, K.R.; *Blood* (1984), **63**, 518.
58. Quinlan, G.J.; Halliwell, B.; Moorhouse, C.P. & Gutteridge, J.M.C.; *Biochim. Biophys. Acta* (1988), **962**, 196.
59. Askira, Y.; Rubin, B. & Rabinowitch, H.D.; *Free Rad. Res. Commun.* (1991), **12-13**, 837.
60. Rebeiz, C.A.; Juvik, J.A. & Rebeiz, C.C.; *Pest. Biochem. Physiol.* (1988), **30**, 11.
61. Goff, B.A.; Bachor, R.; Kollias, N. & Hasan, T.; *J. Photochem. Photobiol. B: Biol.* (1992), **15**, 239.
62. Verma, A.; Kolodgle, F.D.; Hirsh, D.J.; Carlson, G.C.; Virmani, R. & Snyder, S.H.; *Circulation* (1992), **86** (Suppl. I), 757.
63. Moncada, S.; Palmer, R.M.J. & Higgs, E.A.; *Pharmacol. Rev.* (1991), **43**, 109.
64. Sies, H.; *Angew. Chem. Int. Ed.* (1986), **25**, 1058.
65. Halliwell, B. & Gutteridge, J.M.C.; *Free Radicals in Biology and Medicine* , Clarendon Press, Oxford, 1989.

This special issue of Química Nova is dedicated to Prof. G. Cilento on the occasion of his 70th birthday and is financed by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).