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Review

The dual face of endogenous α-aminoketones: Pro-oxidizing metabolic weapons ☆

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Received 31 January 2006; received in revised form 26 June 2006; accepted 6 July 2006
Available online 14 July 2006

Abstract

Amino metabolites with potential prooxidant properties, particularly α-aminocarbonyls, are the focus of this review. Among them we emphasize 5-aminolevulinic acid (a heme precursor formed from succinyl–CoA and glycine), aminoacetone (a threonine and glycine metabolite), and hexosamines and hexosimines, formed by Schiff condensation of hexoses with basic amino acid residues of proteins. All these metabolites were shown, *in vitro*, to undergo enolization and subsequent aerobic oxidation, yielding oxyradicals and highly cyto- and genotoxic α-oxoaldehydes. Their metabolic roles in health and disease are examined here and compared in humans and experimental animals, including rats, quail, and octopus. In the past two decades, we have concentrated on two endogenous α-aminoketones: (i) 5-aminolevulinic acid (ALA), accumulated in acquired (e.g., lead poisoning) and inborn (e.g., intermittent acute porphyria) porphyric disorders, and (ii) aminoacetone (AA), putatively overproduced in diabetes mellitus and *cri-du-chat* syndrome. ALA and AA have been implicated as contributing sources of oxyradicals and oxidative stress in these diseases. The end product of ALA oxidation, 4,5-dioxovaleric acid (DOVA), is able to alkylate DNA guanine moieties, promote protein cross-linking, and damage GABAergic receptors of rat brain synaptosome preparations. In turn, methylglyoxal (MG), the end product of AA oxidation, is also highly cytotoxic and able to release iron from ferritin and copper from ceruloplasmin, and to aggregate proteins. This review covers chemical and biochemical aspects of these α-aminoketones and their putative roles in the oxidative stress associated with porphyrias, tyrosinosis, diabetes, and *cri-du-chat*. In addition, we comment briefly on a side prooxidant behaviour of hexosamines, that are known to constitute building blocks of several glycoproteins and to be involved in Schiff base-mediated enzymatic reactions.
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Keywords: 5-aminolevulinic acid; Aminoacetone; Hexosamines; Oxidative stress; Amino acid metabolism; Diabetes; Porphyrias; *Cri-du-chat* syndrome

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☆ This paper is part of the 4th special issue of CBP dedicated to The Face of Latin American Comparative Biochemistry and Physiology organized by Marcelo Hermes-Lima (Brazil) and co-edited by Carlos Navas (Brazil), Rene Beleboni (Brazil), Rodrigo Stabeli (Brazil), Tania Zenteno-Savín (Mexico) and the editors of CBP. This issue is dedicated to the memory of two exceptional men, Peter L. Lutz, one of the pioneers of comparative and integrative physiology, and Cicero Lima, journalist, science lover and Hermes-Lima's dad.

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doi:10.1016/j.cbpc.2006.07.004

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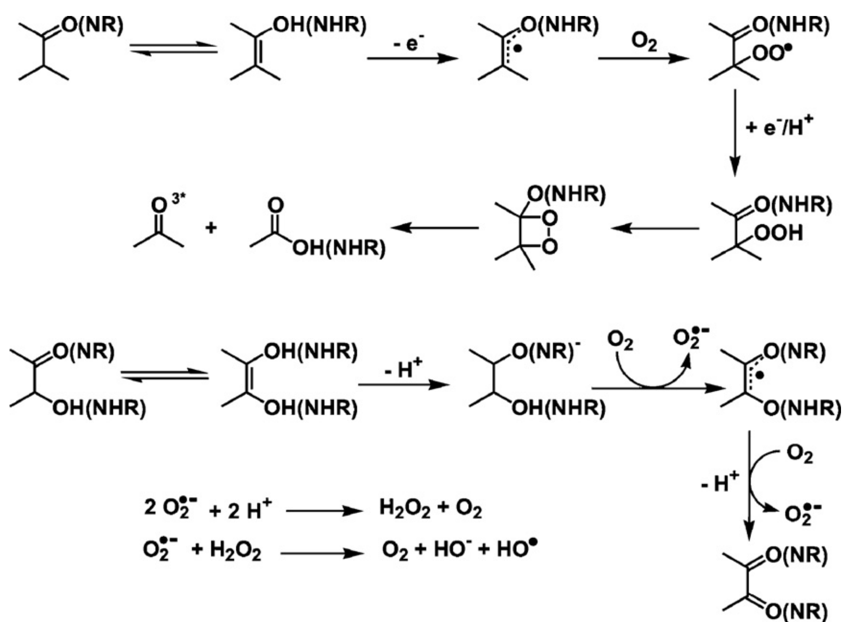
1. Introduction

Life on Earth depends on the capacity of organisms to extract solar and chemical energy from the environment, a complex and yet mysterious play performed by innumerable low and high molecular weight ionic and molecular protagonists. Paradoxical are the dual roles that many pivotal biological species execute, among them: (i) dioxygen — the final electron acceptor in respiration but also a source of life-threatening reactive oxyradicals such as hydroxyl and alkoxyl radicals (Fridovich, 2004); (ii) iron and copper — essential metals for many protein and enzyme activities, but potentially dangerous when "free", i.e., coordinated to small ligands and available for redox cycling coupled with radical production in the presence of peroxides (Welch et al., 2002; Halliwell and Gutteridge, 1999); and (iii) phosphate ions — fundamental for bioenergetics, signal transduction, and metabolite compartmentalization, but also a potential inducer of mitochondrial lipid peroxidation and permeabilization, thereby impairing the cell energy machinery (Kowaltowski et al., 1995). The drama of cell life depends on the right molecules, ions and radicals being in the right places (spatiality/compartmentalization) at the right times (temporality), in a balanced concentration and endowed with an adequate reactivity (rate constants). Disturbances in this delicate balance may culminate in disease and death. In this review, we bring into discussion possible deleterious outcomes of enzyme deficiencies accompanied by accumulation of certain amino-carbonyl metabolites in virtue of their capacity to react with dioxygen in the presence of transition metals yielding reactive species and triggering oxidative stress. We focus mainly on the normal metabolism of amino acids in humans as compared to rats, birds, cephalopods and other animals and emphazise a potential prooxidant role of some amino metabolites, namely 5-aminolevulinic acid (ALA) – a heme precursor accumulated in porphyrias –, and aminoacetone (AA), a threonine catabolite implicated as a source of cytotoxic methylglyoxal (MG) in diabetes mellitus.

Indeed, a large number of carbonyl and phenolic metabolites overproduced in inherited and acquired disorders have been shown to undergo *in vitro* metal-catalyzed oxidation by molecular oxygen, yielding very reactive intermediates and products, among them peroxides, radicals, triplet species and α-oxoaldehydes (Bechara et al., 1995; Thornalley et al., 2000; Royer et al., 2004). Many of these reactions, when studied in the presence of iron and copper storage proteins such as ferritin and ceruloplasmin, were accompanied by metal release and enhanced generation of reactive oxygen (ROS) and nitrogen species (RNS). Iron and copper overload are biological events of medical relevance (e.g., in diabetes, porphyria, lead poisoning, Wilson disease, haemochromatosis, neurodegenerative diseases, and thalasemia, among others) for the redox unbalance they cause in cell homeostasis and organ integrity and functionality (Halliwell and Gutteridge, 1999). The identification of such potentially deleterious reactions are thus of utmost importance as they may offer important clues to understanding the clinical manifestations of several maladies and possibly to designing dietary and pharmacological therapies.

2. Metabolic classes of prooxidants

Endogenous and xenobiotic polyphenols (e.g., homogentisic acid, divicine, hydroanthraquinones, 6-hydroxydopamine) and the enol form of carbonyl metabolites (e.g., ALA, AA, 2-methylacetoacetate, succinylacetone) belong to organic functions expected to promptly donate one electron to dioxygen, especially in slightly alkaline medium containing phosphate ions (Bechara et al., 1995) (Scheme 1). Phosphate ions have been shown to catalyze carbonyl enolization, thus favoring substrate electron transfer to dioxygen (Baader et al., 1985). Semiquinone ($HQ^{\cdot}$) or enoyl ($HE^{\cdot}$ a semiquinoid species) radicals are formed in addition to superoxide anion radicals ($O_2^{\cdot-}$), of which the chemical or superoxide dismutase (SOD)-catalyzed disproportionation of $O_2^{\cdot-}$, yields hydrogen peroxide (H_2O_2). All of the abovementioned radicals – $HQ^{\cdot}$, $HE^{\cdot}$ or $O_2^{\cdot-}$ – reportedly release iron from ferritin (Bolann and Ulvik, 1987; Monteiro et al., 1988; Rocha et al., 2000a). In turn, $HQ^{\cdot}$ and $HE^{\cdot}$ species can (i) undergo dismutation to H_2Q/Q and H_2E/E, respectively, (ii) reduce dioxygen to $O_2^{\cdot-}$, or (iii) reduce $O_2^{\cdot-}$ to H_2O_2. Additionally, $HE^{\cdot}$ radicals can suffer dioxygen insertion yielding an alkylperoxyl radical ($HE–OO^{\cdot}$), that can propagate the aerobic oxidation of the metabolite and form a 1,2-dioxetane intermediate (Bechara et al., 1979). 1,2-dioxetanes are unstable compounds whose fate is principally to undergo thermal cleavage yielding a carbonyl product in the electronically excited triplet state (Kopecky and Mumford, 1969; Bechara and Wilson, 1980; Adam, 1982). The photochemical, alkoxyl-like properties of triplet carbonyls produced either by irradiation or enzyme-catalyzed reactions and the potential risk they represent to cell structures have been solidly established



Scheme 1. Generation of reactive intermediates and products by α-hydroxy and α-aminocarbonyl metabolites.

(Cilento and Adam, 1995, and references therein). On the other hand, the H_2O_2 afforded by $O_2^{\cdot-}$ dismutation or $O_2^{\cdot-}$ reduction by $HQ^{\cdot}$ and $HE^{\cdot}$ is expected to undergo Fenton-type reactions ($Me^{n+}+H_2O_2 \rightarrow Me^{n+1}+HO^{\cdot}+HO^{-}$) with reduced transition metal ions ($Me=Fe^{2+}$, Cu^{1+}) to form extremely oxidizing and potentially dangerous hydroxyl radicals (Halliwell and Gutteridge, 1999). The expected dioxygen-dependent chemistry of the carbonyl metabolites discussed above is summarized in Scheme 1 (Bechara et al., 1995), where prooxidant metabolites are classified into two categories:

Class A: Carbonyl, imino and β-dicarbonyl metabolites, especially when α-substituted by an alkyl group, which undergo enolization followed by one-electron oxidation by several biological oxidants such as hemeprotein/H_2O_2, oxyradicals, and peroxynitrite. Examples: *n*-hexanal (a lipoperoxidation product), isobutanal (a model carbonyl substrate), Schiff bases (several metabolites and intermediates of enzymatic reactions), succinylacetone (SA, a tyrosine metabolite), and 2-methylacetoacetate (an isoleucine metabolite). α-alkylated carbonyl compounds display a lower oxidation potential than the non-alkylated homologues, thus favoring electron transfer to dioxygen. Subsequently, dioxygen insertion in the enoyl $EH^{\cdot}$ radical product yields a peroxyl radical ($EOO^{\cdot}$), a dioxetane intermediate by $EOO^{\cdot}$ cyclization, and ultimately a triplet product by dioxetane homolysis. The latter may decay thermally, emit light (chemiluminescence) or behave like alkoxyl radicals undergoing hydrogen abstraction from hydrogen donors (e.g., polyunsaturated fatty acids), fragmentation to radicals, isomerization, and cycloaddition to unsaturated substrates (Cilento and Adam, 1995).

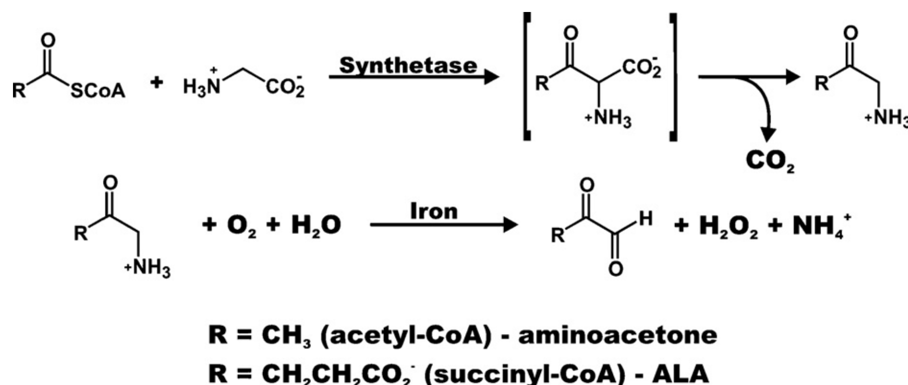
Class B: α-hydroxy and α-amino carbonyl metabolites, whose enolization is followed by electron transfer to dioxygen yielding radical and a resonance stabilized enoyl radical ($EH^{\cdot}$). Subsequently, $EH^{\cdot}$ may transfer a second electron to dioxygen, producing an α-dicarbonyl or an α-iminocarbonyl compound plus $O_2^{\cdot-}$ radical. This is the case of triose phosphates, hexosamines, ALA (accumulated in some porphyrias), and AA (overproduced in diabetes) (Mashino and Fridovich, 1987; Monteiro et al., 1989; Dutra et al., 2001). α-dicarbonyls produced either directly or from α-iminocarbonyl hydrolysis by these reactions, such as biacetyl, MG, and 4,5-dioxovaleric acid (DOVA), have been shown to be highly cytotoxic and genotoxic (Abordo et al., 1999; Douki et al., 1998).

Iron, hydroxyl radical and α-dicarbonyl overloads have been implicated in a plethora of inherited and chemical disorders, including mutagenesis, cancer, aging, diabetes, neurodegenerative processes, several metabolism errors, inflammatory diseases, pollution- and smoking-associated diseases, and heavy metal poisoning. Far more difficult has been to demonstrate the intermediacy of triplet carbonyl species in normal and pathological processes ("photochemistry without light"), as long proposed by White et al. (1969) and Cilento (1974).

3. α-aminoketones

Among other caracteristics, α-aminoketones differ from primary amines for their property of undergoing rapid enolization in physiological pH and, in an aerated medium, subsequent oxidation by molecular oxygen via superoxide radical intermediate, forming α-oxoaldehydes and H_2O_2 (Bechara, 1995). The biochemical and medical literature of the last three decades highlight the crucial roles of these reactive species in the cellular metabolism as chemical mediators or signalers of numerous normal and deleterious processes (Halliwell and Gutteridge, 1999); hence the importance of unveiling the prooxidant role of α-aminoketones in cells.

Our research group became interested in the chemistry of endogenous aminoketones 20 years ago while studying the mechanism of iron-catalyzed ALA oxidation by molecular oxygen (Bechara et al., 1993). ALA is the first metabolite of the



Scheme 2. Biosynthesis of aminoacetone (AA) and 5-aminolevulinic acid (ALA) and subsequent oxidation to α-oxoaldehydes.

biosynthetic pathway of the heme group, the prosthetic group of key proteins and enzymes for the aerobic life such as myoglobin, hemoglobin, chlorophyll, cytochromes, catalase, and many dioxygenases. It accumulates in the blood and other tissues (mainly the liver and brain) of patients with hereditary (e.g., intermittent acute porphyria and tyrosinemia) and acquired (e.g., lead poisoning) porphyria, where it triggers serious tissue and neurological damage. ALA oxidation has been shown *in vitro* to yield DOVA, NH_4^+ ions and ROS by metal-catalyzed oxidation by dioxygen (Scheme 2). The end product of ALA oxidation, DOVA, presumably possesses important genotoxic activity, for it is able to form cyclic adducts in its reaction with residues of DNA adenine and guanine (Douki et al., 1998).

Another aminoketone we have studied in recent years is AA (Dutra et al., 2001, 2003), a threonine and glycine catabolite that accumulates in syndromes such as *cri-du-chat* (Kuhner et al., 1974) and threoninemia (Reddi, 1978), diseases characterized by a high level of circulating threonine. Threonine has recently been reported as a ketogenic amino acid in quail (*Coturnix coturnix japonica*) liver, especially while fasting, which fits the use of triacylglycerol and ketone bodies as fuel by migratory birds, whereas it is glucogenic, via the intermediacy of AA, MG, and D-lactate, in rat liver and octopus (*Octopus vulgaris*) tentacles (Akagi et al., 2004; Akagi and Ohmori, 2004). AA has also recently been identified, together with triose phosphates and acetone, as an endogenous source of MG in diabetes mellitus (Kalapos, 1999a,b) (Scheme 2). MG is a potent protein and DNA modifying agent that accumulates mainly in tissues susceptible to lesions observed in diabetes — retina, kidneys and nerves (Kalapos, 1999a,b). The discovery of the endogenous production of AA by bacteria and mammals occurred concomitantly with the growing interest in aminoketones, triggered by the discovery of ALA and its principal biological function — the biosynthesis of heme. In the late 1950s, Gibson et al., 1958 demonstrated that isolated particles of chicken (*Gallus gallus*) reticulocytes were able to synthesize ALA from succinyl–CoA and glycine, and AA from acetyl–CoA and glycine. The reactions involved in the biosynthesis of ALA and AA are essentially the same (Scheme 2).

The α-hexosamines, derived from hexoses by substitution of the 2-hydroxyl group by an amine group, also belong to the class of the endogenous α-aminoketones and are thus expected to act as prooxidants. Indeed, their aerobic oxidation also produces H_2O_2 and α-oxoaldehydes, according to Hiraku and Kawanishi (1999). Recently, α-hexosamines have been related with the metabolic control of the secretion of leptin, a signalling protein of satiation, and with a possible nutritional "sensor" role (Brownlee, 2001; Laferrère et al., 2004).

Regarding the end products of α-aminoketone oxidation, α-dicarbonyl catabolites, Szent-Györgyi (1941, 1968, 1973) hypothesized long ago that they could participate in deleterious biological processes, particularly in aging (Szent-Györgyi, 1941, 1968; Kalapos, 1999a,b), based on their electronic configuration and high reactivity. For example, formation of Schiff bonds between each of the carbonyl groups of the α-dicarbonylic compound and the amine group of proteins may cause protein aggregation and denaturation by cross-linking (Prot–N═CR–CR═N–Prot). Another important biological consequence of Schiff's reaction is the formation of fluorescent adducts called Advanced Glycation End products (AGEs), or Maillard products, such as pentosidines (Monnier, 1989; Singh et al., 2001; Thornalley, 2002; Cameron et al., 2005; Monnier et al., 2005). Pentosidines are putative aging markers formed by cross-linking between lysine and arginine protein residues and oxidized pentose and other sugars (Fig. 1). The AGEs are known for their ability to attach to specific cellular receptors, triggering various events such as the induction of cytokines and growth factors (Vlassara et al., 1988; Kirstein et al., 1990),

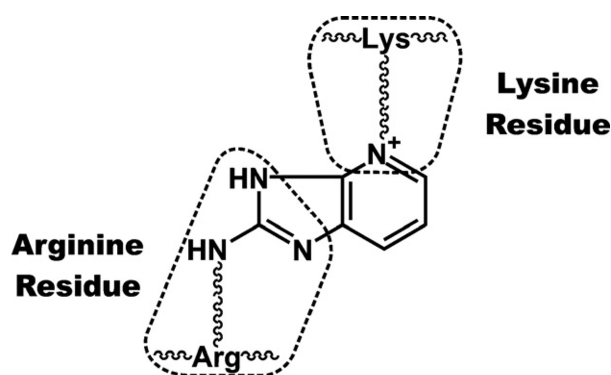


Fig. 1. Pentosidine, an advanced glycation end product (AGE) formed from a pentose and lysine and arginine residues of proteins.

oxidative stress (Yan et al., 1994), and the regulation of cellular adhesion (Vlassara et al., 1995; Schmidt et al., 1995).

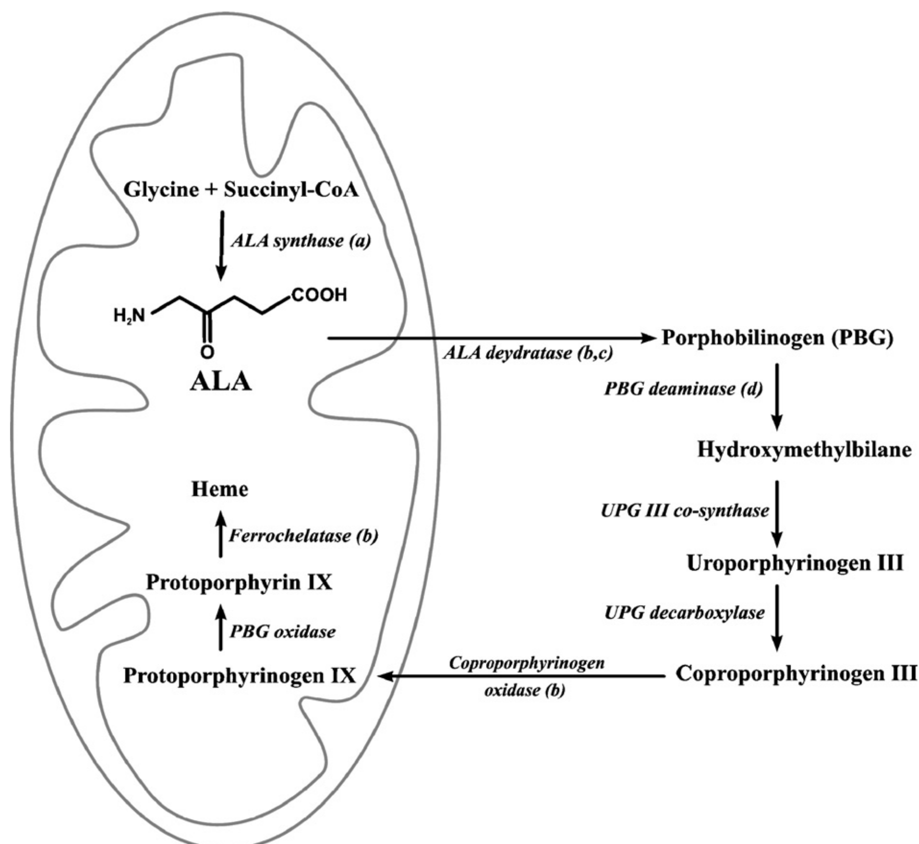
4. 5-aminolevulinic acid (ALA) metabolism and porphyria

Porphyrias are metabolic disorders associated with enzymatic insufficiency or inhibition of the biosynthetic pathway of the heme group (Scheme 3), leading to the accumulation of ALA and/or porphyrin intermediates in tissues and the urinary or fecal excretion of this excess (Hindmarsh, 1986; Kauppinen, 2005). The porphyrias have been clinically grouped into erythropoietic and hepatic types, depending on whether the excessive production of the intermediaries occurs primarily in the bone marrow or in the liver, triggering, respectively, acute or chronic skin and neurovisceral symptoms.

Hereditary hepatic porphyrias include (i) intermittent acute porphyria (IAP), which is attributed to the deficient biosynthesis of porphobilinogen (PBG) deaminase (Hindmarsh, 1986), and (ii) hereditary tirosinemia, where strong competitive inhibition of ALA dehydratase (K_i = 0.03 μM) by a tyrosine catabolite, SA, occurs (Kappas et al., 1989). SA, in turn, accumulates in tyrosinemia patients due to deficient biosynthesis of fumarylacetoacetate hydrolase of the tyrosine degradation pathway. Among acquired porphyrias, lead poisoning (also named plumbism and saturnism) stands out for its multifactorial biochemical and biological effects, attributed primarily to the inhibition of ALA dehydratase and iron chelatase by Pb^{2+} ions and leading, respectively, to ALA and protoporphyrin IX overload (Stohs and Bagchi, 1995; Gurer and Ercal, 2000). In all these cases, high ALA concentrations can be measured in the urine, blood and other tissues. Relevant biochemical, environmental and sociological aspects of lead poisoning will be discussed in another section of this review.

Accumulation of ALA and PBG in cellular fluids and in tissues of IAP patients has long been known to be closely related with the clinical symptoms (Stein and Tschudy, 1970; Gorchein, 1984). Many forms of impairment of the peripheral and central nervous system have been observed, including neuron demyelinization and vacuolization (Gibson and Goldberg, 1956; Cavanagh and Mellick, 1965; Becker and Kramer, 1977; Anzil and Dozic, 1978). In addition, a high incidence of primary liver cancer in IAP and hereditary tyrosinemia carriers has been associated with the frequency of acute attacks, when plasmatic ALA levels reach about 100 times the normal value (~0.26 μM) (Douki et al., 1998; Lithner and Wetterberg, 1984; Gubler et al., 1990; Thunnissen et al., 1991). A carcinogenic potential of ALA was inferred from its ability to promote *in vitro* and *in vivo* DNA guanine oxidation to 8-oxo-dihydro-2′-deoxyguanosine (8-oxodG) and from the detection of DOVA adducts with DNA bases (Douki et al., 1998).

ALA undergoes enolization and subsequent metal-catalyzed aerobic oxidation mediated by superoxides with the production



Scheme 3. Biosynthetic pathway of the heme group. Deficiencies in the heme biosynthetic pathway leading to 5-aminolevulinic acid (ALA) overload: (a) negative feedback inhibition by heme; in acute intermittent porphyria (AIP) patients, activated by certain drugs and metabolites; (b) inhibited by lead (lead poisoning); (c) inhibited by succinylacetone (SA) (hereditary tyrosinemia); (d) deficient biosynthesis in AIP.

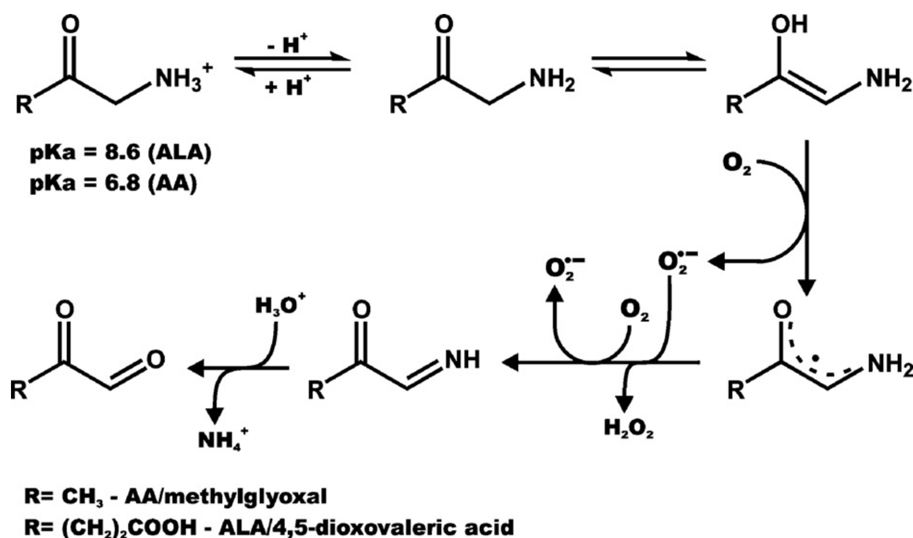
of H_2O_2 and, hence, generation of reactive $HO^{\cdot}$ radicals via Fenton reaction (Monteiro et al., 1989; Bechara, 1996). Interestingly, not only α-amino (e.g., ALA and AA) but also α-hydroxy (e.g., dihydroxyacetone and glucose) carbonyl compounds undergo enolization in aqueous and organic media (Mashino and Fridovich, 1987; Thornalley, 1985; Dutra et al., 2001) and thus are expected to behave chemically like polyphenols (e.g., homogentisic acid) and aminophenols (e.g., divicine), yielding ROS in their reaction with dioxygen (Martin and Batkoff, 1987; Chevion et al., 1982; Bechara, 1995) (Schemes 1 and 2). Over the last two decades, Bechara and coworkers demonstrated, both *in vitro* and *in vivo*, that ALA-generated ROS and DOVA are capable to damage hemoglobin, ferritin, DNA, synaptosomes and mitochondria, among other biomolecules and organelles. These results are underpinned by the ALA oxidation mechanism depicted in Scheme 4.

After enolization, ALA undergoes oxidation by dioxygen with the formation of the $ALA^{\cdot}$ enoyl radical, while oxygen is reduced to the $O_2^{\cdot-}$. Both $O_2^{\cdot-}$ and $ALA^{\cdot}$ can propagate the reaction free radical chain. Further oxidation of $ALA^{\cdot}$ by dioxygen produces $O_2^{\cdot-}$ and ALA-derived imine (ALA_{imine}) which, after hydrolysis, produces DOVA and NH_4^+ ion in stoichiometric amounts relative to the O_2 consumed. Hydrogen peroxide, formed by $O_2^{\cdot-}$ dismutation and by $O_2^{\cdot-}$ reduction by ALA and $ALA^{\cdot}$, subsequently reacts with iron, yielding $HO^{\cdot}$ radicals (Fenton reaction). Hydroxyl radical production by ALA was clearly demonstrated both *in vitro* (Monteiro et al., 1989) and *in vivo* (Timmins et al., 1999), by EPR spin trapping techniques with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), respectively.

DOVA is the end product of ALA oxidation and is also accumulated in porphyrias. DOVA, as well as various carbonyl toxicants such as malondialdehyde, acrolein, 2,4-decadienal, acetaldehyde and MG, is endowed with the potential of alkylating DNA bases (O'Brien et al., 1989; Balu et al., 2002; Onuki et al., 2003). Accordingly, Douki et al. (1998) and Cadet et al. (1999a) reported efficient guanine alkylation of both guanosine and isolated DNA promoted by DOVA. They suggested that the first stage of DNA alkylation involves reversible formation of a Schiff base between the N^2-amino group of 2′-deoxyguanosine (dGuO) and the DOVA aldehyde group. Subsequently, a secondary attack of the N^1 imine group of the guanine moiety on the aldehyde function of DOVA renders a stable five-membered ring guanine-DOVA cyclic adduct.

ALA oxidation has been shown to be particularly damaging in the brain, muscles and liver of rats treated with ALA or the succinylacetone methyl ester (SAME) and may thus explain a wealth of data describing protective enzymatic antioxidant responses (e.g., elevated SOD, catalase and glutathione peroxidase) in AIP and lead poisoned patients and the effectiveness of coupled chelator and antioxidant therapy of lead poisoning. These effects are summarized in Fig. 2 and briefly described below:

1. *In vitro*, ALA has been proven to cause oxidative injury to proteins, liposomes, mitochondria, synaptosomes and DNA, as well as release of ferritin iron and activation of the iron regulating protein IRP-1 (Monteiro et al., 1989; Hermes-Lima et al., 1992; Oteiza and Bechara, 1993; Fraga et al., 1994; Onuki et al., 1994; Carvalho et al., 1997).
2. ALA or SAME administered to rats mobilizes iron to the liver and brain, degrades mitochondria in liver, soleus and gastrocnemius, peroxidizes membranes of various tissues, and induces the biosynthesis of two main antioxidant enzymes, namely SOD and glutathione peroxidase (GPX) (Pereira et al., 1992; Rocha et al., 2000a,b, 2003).
3. Effects of ALA on rat and human CNS were explained by ALA binding to receptors of the neurotransmitter γ-aminobutyric acid ($GABA_A$) (Brennan and Cantrill, 1979), followed by receptor oxidative lesions promoted by ALA-generated oxyradicals and DOVA (Demasi et al., 1996; Penatti et al., 1996).
4. Symptomatic AIP carriers, treated or not with hematin, possess 2-fold increased blood SOD and GPX activities, whereas SOD is only 30% higher in latent carriers (Medeiros et al., 1982).



Scheme 4. Non-enzymatic aerobic oxidation of α-aminoketones.

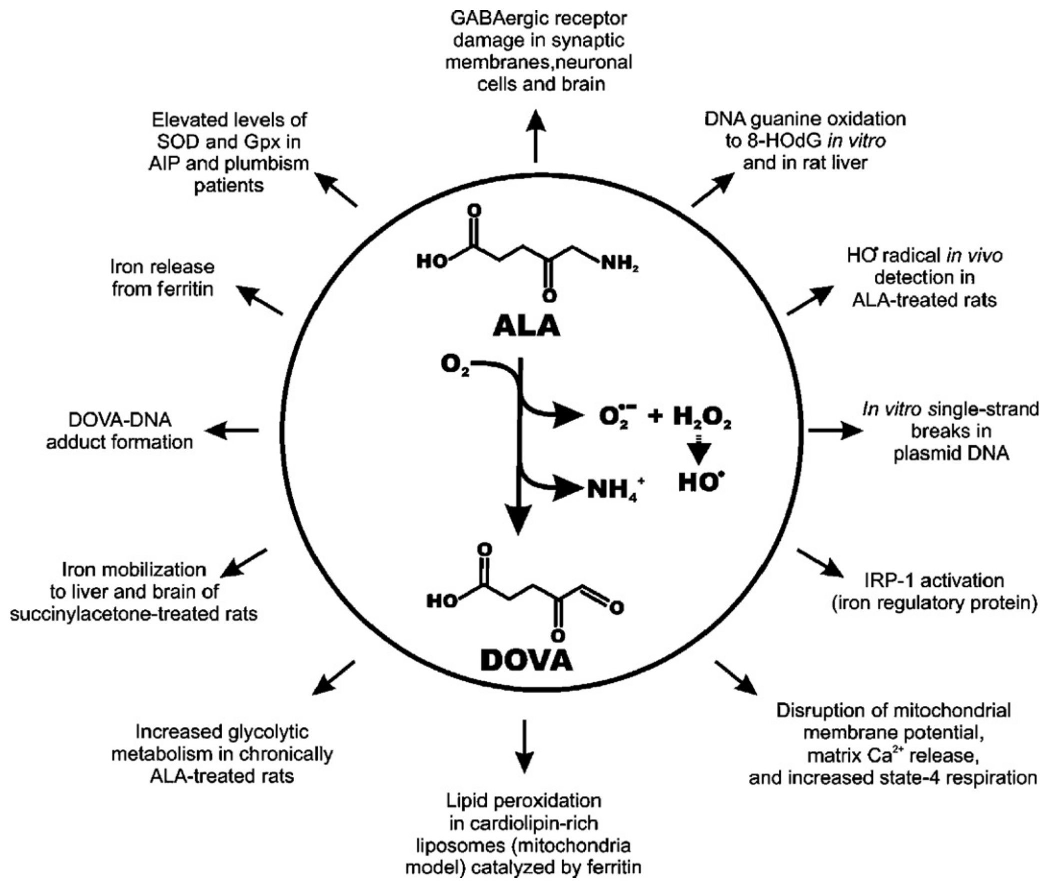
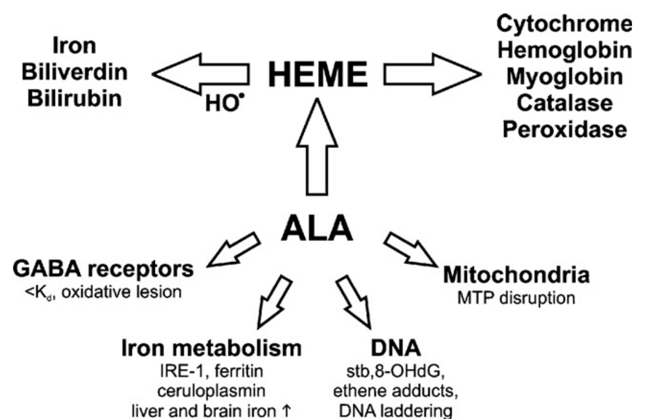


Fig. 2. Oxidative damage to biomolecules and cells promoted by 5-aminolevulinic acid (ALA).

5. The frequency of elevated SOD in lead-exposed workers increases exponentially above 30 μg/dL blood and is positively correlated with putative biological markers of oxidative stress such as methemoglobin and urinary chemiluminescence (Monteiro et al., 1985; Costa et al., 1997a,b).
6. Several authors have proposed the use of antioxidants associated with chelators to remediate the clinical manifestations of lead poisoning (Gurer and Ercal, 2000; Hsu and Guo, 2002).

The methyl ester of succinylacetone, SAME, is more diffusible across membranes than the corresponding acid, SA, and is therefore an adequate drug to establish a porphyric state in experimental animals. Accordingly, upon *i.p.* SAME administration to rats, the observed plasma, liver and urine ALA concentrations increased with the drug dosage (10–80 mg/kg body weight) (Bechara and coworkers, unpublished). As expected, HPLC analyses of urinary porphyrins of SAME-treated rats displayed decreased levels of porphyrins, especially coproporphyrin, which is known to be down-regulated by ALA accumulation.

Because ALA occupies a central and critical place on the map of aerobic metabolism, acting as the precursor of the porphyrins incorporated in cytochromes, hemoglobin, myoglobin, many peroxidases, and chlorophyll, as well as a source of ROS by iron-catalyzed oxidation capable of damaging proteins, membranes and DNA (Scheme 5), it is reasonable to propose a role of ALA in the aging process. Accordingly, we have recently found that the blood ALA concentration in nine- and sixteen-month-old rats increases two- and seven-fold, respectively, when compared with two-month-old rats (Dutra and Bechara, unpublished) (Table 1). Important ALA increments and iron mobilization were also observed in the liver of old rats



Scheme 5. Metabolic fates of 5-aminolevulinic acid (ALA): prooxidant and antioxidant roles. After Ryter and Tyrrel (2000), with additions from Dutra et al. (2003) and De Siervi et al. (2002).

Table 1
Plasma and liver 5-aminolevulinic acid (ALA) increases with age

	Age	Plasma (nM)	Liver (nmol/mg protein)
Rats ($n=10$)[a]	2 months	16	<4
	9 months	53	18
	16 months	106	57
Humans	Children[b]	80	–
	Adults	260[c], 300[d], 3500[e]	–

[a] Dutra and Bechara, unpublished; [b] Sithisarankul et al., 1999; [c] Costa et al., 1997a,b; [d] Murata et al., 2003; [e] Minder, 1986.

The scarce and inaccurate data in the literature on human blood ALA also show a trend for elevated ALA in aged individuals (4- to 40-fold) as compared with children.

When challenging ferritin with ALA, we observed that not only ALA-generated $O_2^{\cdot-}$, but also the resulting $ALA^{\cdot}$ enoyl radical can reduce ferritin iron(III) to iron(II) and release iron from ferritin (Rocha et al., 2003). Moreover, this process was accompanied by oxidative damage of tryptophan and cysteine residues of the L and H subunits of ferritin exposed to ALA, possibly explaining the observed loss of iron uptake activity by ferritin. An expected consequence of this process, if operative *in vivo*, is iron overload and exacerbated oxidative stress. Concomitantely, we found significant decrements in antibody recognition of different human ferritins, mainly those from spleen, liver and brain. In this context, it is worth recalling the impairment of the immunological and neurological systems of lead poisoned individuals and the high frequency of primary liver cancer in patients suffering from IAP and tyrosinosis.

Also noteworthy in the context of redox imbalance driven by ALA are the ALA-based photodynamic herbicide (Tripathy and Singhal, 1999) and insecticide (Sasikala et al., 1994) formulations and the use of ALA and its esters (Ackroyd et al., 1999; Casas et al., 2001; Luksiene et al., 2001; Gerscher et al., 2001) in photodynamic therapy (PDT) of cancer, where ALA acts as an endogenous generator of photosensitizing porphyrins (Oseroff, 1993; Ackroyd et al., 1999).

PDT is a current treatment of choice against tumors, either singly or in combination with chemotherapeutical, immunological, surgical or radiological approaches. It is based on the photodynamic action of light, dioxygen and a photosensitizer, an efficient source of cytotoxic singlet oxygen and free radicals that are able to destroy tumoral cells (Weishaupt et al., 1976). In these processes, photon absorption by the sensitizer (a pigment) excites this molecule to the singlet (fluorescent) state, which is followed by intersystem crossing to the triplet (phosphorescent) state, and subsequently by energy transfer to the colliding ground (triplet) state oxygen molecule to yield excited (singlet) oxygen or superoxide anion radical (Type II photosensitized oxidations, according to Foote, 1991). Competitive with energy transfer, electron transfer from the triplet pigment to the substrate or the solvent, forming reactive ion radicals, may also occur depending on several factors such as reduction potentials of the reagents, solvent polarity, and local oxygen concentration (Type I photosensitized oxidations). Recently, Redmond and Gamlin (1999) compiled the quantum yields (Φ) of singlet oxygen formation – singlet oxygen molecules/photons absorbed – by several photosensitizers, including methylene blue (0.76), mesochlorin (0.79), zinc protoporphyrin IX (0.91), rose bengal (0.79) and Photofrin® (a mixture of hematoporphyrins) (0.89).

PDT based on ALA (Levulan®), as an endogenous source of porphyrins, has recently been employed, either topically, orally, internally or intravenously, in the treatment of superficial and nodular basal cell carcinoma, Bowen's disease, actinic keratoses, T cell lymphoma, ectocervical dysplasia and associated papilloma virus infections, vulvar intraepithelial neoplasia, superficial head and neck cancer, gastrointestinal cancers, dysplastic Barrett's esophagus, superficial urothelial cancer of the bladder, psoriasis, endometrial ablation, and noduloulcerative basal cell carcinomas (Lang et al., 2001; Marcus and McIntyre, 2002; Pech et al., 2005; Britton et al., 2005; Babilas et al., 2005). However, PDT with ALA has also been found to cause undesirable side effects on healthy cells (Wolfsen and Ng, 2002) and hepatic dysfunctions (Fuchs et al., 2000; Duez et al., 2001), which may be attributed to the prooxidant properties of ALA as a source of oxyradicals (Bechara, 1996). Accordingly, ALA-injected swimming-trained rats underwent a notable shift to glycolytic metabolism when compared with non-treated controls (Pereira et al., 1992). This was indicated by severe muscle and hepatic mitochondrial degeneration and consequent losses in glycogen, citrate synthase and Mn–SOD levels, and by concentration increases in blood free fatty acid and lactate. The ALA-treated rats were able to swim for only about half the average endurance time recorded for non-treated animals (40 *versus* 90 min).

The remarkable photodynamic properties of ALA disclosed by recent advances in cancer PDT led many investigators to develop ALA-based pesticides, herbicides, and plant growth regulators (Sasaki et al., 2002; Fukuda et al., 2005, and references therein). A growing number of patents on chemical, enzymatic, and bacterial manufacture of ALA and biodegradable ALA derivatives and their applications in agriculture have been registered in the past decade. Noteworthy are inventions related to the production of transgenic plants such as maize with a modified ALA synthase function, that is resistant to the treatment with herbicides based on a glutamate-1-semialdehyde aminotransferase inhibitor, whereas weeds are not.

5. ALA and lead poisoning

Since ancient times, lead poisoning has afflicted millions of people all over the world, both in developed and poor nations. It is an ubiquitous and insidious toxin present not only in air, dust, water, soil, food and materials like paints, car batteries, and gasoline, but also hidden in ceramic dishes, crystal baby bottles, herbal medicines, moonshine, and eye make-up. Lead has long been known to be highly toxic to several organs, especially the brain and kidneys, and its main victims are children and lead-exposed workers (Lidsky and Schneider, 2003; Bellinger, 2004). In the USA, the population of children with blood lead above 100 µg/L has declined by over 80% since leaded gasoline, lead solder in canned foods, and lead in house paint and other products, including toys, was banned. Recent studies

have revealed that chronic, low exposure of children to lead, leading to blood levels as low as 50 μg/L, is enough to trigger learning disabilities, attention and memory deficits, low IQ, aggressiveness and delinquent/criminal behavior (Chisolm, 2001; Koller et al., 2004; Needleman, 2004; Shannon et al., 2005). Considering lead is an invisible, cumulative, slow-acting, and dangerous toxin, public heath and environmental agencies of several countries established regulations and recommendations of tolerable levels of lead to prevent poisoning. For example, from the U.S. Environmental Agency Protecion and the Centers for Disease Control and Prevention: air: <1.5 μg lead/cubic meter; drinking water: <15 μg/L; paints: $<0.06\%$; blood: <100 μg/L. They also recommend the distribution of pamphlets on lead and lead poisoning prevention in homes, schools and factories.

Domestic and wild fauna have also been reported to be suscetible to intoxication by lead. The former group includes cow, horse, dog, buffalo, goat, sheep, and goose, which were either raised in the vicinity of areas previously contaminated by lead such as non-ferrous metal smelters and car battery plants, or ingested lead shots or pellets (see, for example: De Francisco et al., 2003; Dwivedi et al., 2001; Sharpe, 2004). Among free living animals, raptors like eagles, hawks, vultures, and condors frequently fall victim of ingestion of bullet fragments in carcasses (see, for example: Meretsky et al., 2000; Clark and Scheuhammer, 2003; Pain et al., 2005). The literature on lead poisoning of wild and domestic fauna often deals with determination of the metal in tissues, hemolytic anaemia triggered by lead, and impairment of physiological functions. Not many authors studied biochemical changes associated to oxidative stress triggered by lead in animals, but rats and mice, although it is recognized that immunosuppression, cataractogenesis, infertility, thyroid dysfunction and hypertension caused by lead to these animals may be associated with oxidative stress (Mateo et al., 2003; Avkin-Burns et al., 2003). These authors found increased concentrations of *n*-6 polyunsatuyrated fatty acids in the liver and brain of lead-exposed mallard ducks (*Anas platyrhynchos*), probably connected with increased lipid peroxidation in liver, although without important histopathological changes, and decreased plasma alkaline phosphatase activity that may reflect altered bone metabolism in the ducks. Beyer et al. (2004), when studying contamination of wild birds by heavy meals (Pb, Cd, Zn) in the Tri-State Mining District (OK, KS, MO) of the USA, observed 50% lower activities of the lead-sensitive enzyme ALA dehydratase in red blood cells of robins (*Turdus migratorius*) and cardinals (*Cardinalis cardinalis*), which necessarily promotes accumulation of prooxidant ALA and oxidative stress. Also in accordance with the hypothesis that lead causes oxidative stress, Mousa et al. (2002) reported increased antioxidant status in plasma of lead-treated goats, as attested by significant increases of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, catalase), total thiol groups and lipid peroxides.

A plethora of data points to redox imbalance as a result of lead poisoning (Monteiro et al., 1985; Hermes-Lima et al., 1991; Stohs and Bagchi, 1995; Bechara, 1995; Costa et al., 1997a; Gurer and Ercal, 2000; Ercal et al., 2001; Hsu and Guo, 2002). Among the redox-related events triggered by lead, the above quoted authors mention: (i) alteration of membrane fatty acid composition and potentiation of lipid peroxidation; (ii) depletion of glutathione; (iii) inhibition of various thiol proteins and enzymes (e.g., Na^+K^+ ATPase, PKC) by forming mercaptides; (iv) binding to calcium and zinc protein "fingers" and random hydrolysis of nucleic acids; (v) enhanced autoxidation of hemoglobin; (vi) inhibition of antioxidant enzymes; and (vii) accumulation of ALA and protoporphyrin-IX during heme biosynthesis due to inhibition of ALA dehydratase and ferrochelatase, respectively.

Brennan and Cantrill (1979) based on the structural similarity between ALA and GABA, proposed that the neuropsychiatric manifestations of intermittent acute porphyria and lead poisoning, where aminolevulinate accumulates and is excreted, might result from a competition between ALA and GABA for GABAa binding sites of synaptic membranes. Knowing that ALA undergoes prompt enolisation yielding an enolenamine known to be easily oxidisable, we hypothesized that the binding of ALA to GABAa receptors may be followed by in situ ALA oxidation (Demasi et al., 1996).

Undoubtedly, one of most dramatic consequences of lead poisoning is the impairment of neurological functions, especially those involving the cognitive functions and social behavior of children (Chisolm, 2001). The basic mechanisms and clinical correlates of lead-induced neurotoxicity are highly complex and are not discussed in depth here. Lidsky and Schneider (2003) recently reviewed the molecular bases of lead neurotoxicity, in which they implicated apoptosis, excitotoxicity, neurotransmitter storage and release, mitochondria, second messengers, neurotransmission, effects on glia, and, finally, suppression by ALA of GABA-mediated neurotransmission. They emphasized the ability of lead to substitute for calcium, and perhaps zinc, as a factor common to many of its toxic actions. Knowing that ALA behaves as a prooxidant of several biomolecules and supramolecular cell structures, we challenged synaptosomes isolated from rat brain with ALA and examined synaptic membranes obtained from ALA-treated rats for their GABA binding properties (Demasi et al., 1996). Under prolonged treatment of rats with ALA, significant increases were found in non-heme iron, ferritin, and protein carbonyls in the cortex and striatum. In parallel, we demonstrated, *in vitro*, that ALA induces calcium uptake by synaptosomes, leading to permeabilization of their mitochondria. A two-fold decrease in the affinity of GABA receptors for muscimol, a GABA agonist, was induced in rats by ALA-treatment. Altogether, these data pointed to ALA-driven oxidative injury to GABAergic sites of the rat brain preparations and therefore raise a relevant argument in favor of the contribution of ALA-generated reactive oxygen species for neurotoxicity in lead poisoning. Recently, we performed studies with rat brain synaptosomes and neuronal cell lines exposed to ALA and DOVA and also with brains of rats treated with ALA and SAME. A decrease in the density of GABAa receptors in various GABAergic nuclei in the CNS was observed in both ALA and SAME-treated rats. ALA, as already mentioned, reduced the affinity (2-fold higher dissociation constant) of the agonist muscimol for GABAa receptors in

ALA-treated synaptic membranes. Exposure to DOVA caused a 50% decrease in the synaptosomal concentration of GABAergic sites in synaptic membranes, as well as increased rates of cell mortality (Bechara and co-workers, unpublished). In this context, it is worth mentioning that damage on the nervous system of fish (Baatrup, 1991) and *Drosophila* larvae (Morley et al., 2003) exposed to lead has been demonstrated. Considering that lead intoxication is known to induce ALA accumulation, and that ALA damages GABAergic receptors, it is indeed tempting to hypothesize that these effects in lead poisoned animals may be due, in part, to ALA-driven toxicity.

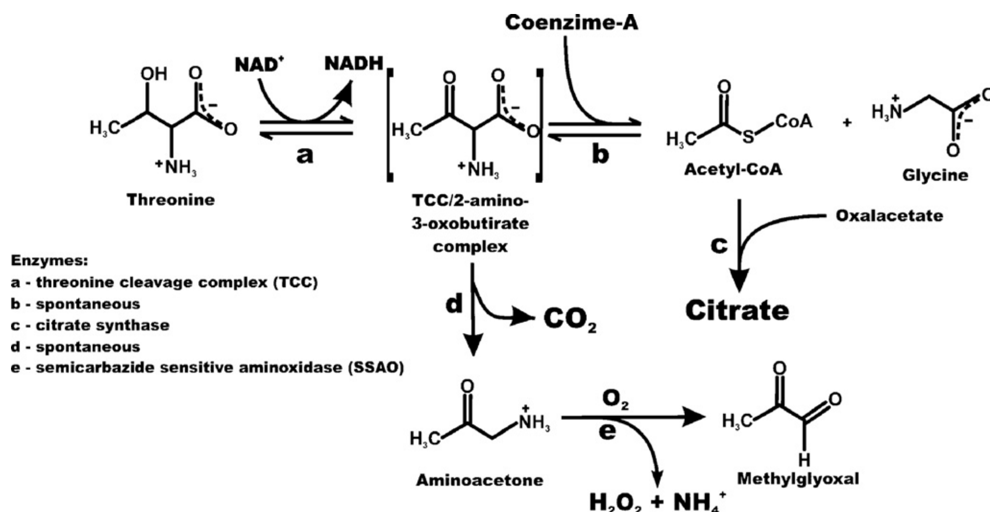
The recognition that lead-induced oxidative stress contributes to the pathogenesis of lead poisoning led several groups to test, in experimental animals and/or humans, lead chelators, anti-oxidants or a combination of both, to minimize the adverse effects of this metal (Gurer and Ercal, 2000; Hsu and Guo, 2002). Some of the results were highly convincing while others were very modest. Some of the chelators and anti-oxidants used were: $CaNa_2EDTA$, Succimer®, penicillamine, and dimercaprol® (chelators) and zinc, selenium, vitamin B6, vitamin C, vitamin E, *N*-acetylcysteine (NAC), taurine, ethoxyquin, α-lipoic acid, captopril, catechins, β-carotene, and melatonin (antioxidants). Chelation therapy of children seriously poisoned by lead has been recommended to be employed with EDTA or succimer when Pb is in the range of 400–700 μg/L blood, and with BAL and CaEDTA when Pb > 900 μg/L blood (Mortensen and Walson, 1993).

A possible connection between lead poisoning and the delinquent behavior of teenagers has been established by several authors. Dietrich et al. (2001) monitored the blood lead levels of 300 teenager residents of Cincinnati (OH, USA), and found that prenatal lead exposure was associated with parent's reports of delinquency and aggression, and postnatal exposure was associated with self-reported acts of violence, which were 4-fold more frequent in youths with Pb > 150 μg/L than in those with Pb < 50 μg/L. In Pittsburgh (PA, USA), using K-line X-ray fluorescence spectroscopy to evaluate the tibia lead levels of 194 arrested and convicted juvenile delinquents, Needleman et al. (2002) found a 4-fold increased risk of delinquency associated with the bone lead level as compared with non-delinquents. Also, Stretesky and Lynch (2004) found that the air-lead levels have a direct effect on property and violent crime rates across 2772 USA counties investigated, even after adjusting for general levels of air pollution and several structural covariates of crimes. If these correlations are unequivocally confirmed in other countries with different socioeconomic and cultural realities, they will convey an important message to the authorities responsible for child education and public health and safety. This message will be that programs and strategies should be designed and applied to prevent the exposure of mothers and children to lead.

6. Threonine/aminoacetone (AA) metabolism

The first researcher to attempt to demonstrate that AA is a threonine catabolite was Elliott (1960a), working with suspensions of *Staphylococcus aureus* and paper chromatography/melting point to identify the reaction products. Probably even the high reactivity of AA — cyclic bimolecular condensation, oxidative deamination and condensation with aldehydes and basic amino acids, its synthesis and its spectroscopic identification were reported only in 1976 (Hepworth). Nevertheless, AA remained commercially unavailable for decades and many authors failed to detect it in biological samples (Urata and Granick, 1963). Only recently a reliable HPLC method for the detection and quantification of AA in biological materials has been described (Kazachkov and Yu, 2005). AA was trapped with a fluorescent label, 9-fluorenylmethyl chloroformate from mouse tissue homogenates, and analyzed by HPLC. Urinary excretion of 20–30 μg AA/mouse/day and about 0.5 μg AA/g liver or small intestine were found by the authors. The physiological and pathological concentrations of AA in human plasma and tissues are yet to be determined.

S. aureus was also found by Elliott (1960a,b) to metabolize glycine to AA, which led the authors to propose two distinct routes for AA formation, starting from either threonine or glycine. In Elliot's studies, three enzymes catalyzing the degradation of threonine were identified: (i) a cytosolic threonine dehydratase that converts AA to 2-oxobutyrate and NH_4^+; (ii) threonine aldolase, which yields acetaldehyde and glycine, also in the cytosol; and (iii) threonine dehydrogenase, a source of 2-amino-3-oxobutyrate in the mitochondria (Scheme 6). Bird and Nunn (1983) estimated that the activities of these three key enzymes in the liver of well-fed rats normally mobilize, respectively, 10%, 3% and 87% of hepatic threonine. However, based on studies carried out with mammals, birds, and marine invertebrates in distinct nutritional states, it is very clear today that the relative contributions of the threonine alternative catabolic routes are affected by species differences, stage of maturity, and modeling approaches (House et al., 2001). For example, Akagi et al. (2004) reported that the activity of threonine aldolase in the livers of rat and quail (*Coturnix coturnix japonica*) is so low that its role in threonine metabolism seems negligible and that threonine is predominantly a ketogenic amino acid in quail, while it is mainly glucogenic in rat. According to these authors, since migratory birds utilize triacylglycerols and ketone bodies as fuel, it is fitting that threonine is oxidized via the ketogenic route, especially while fasting. In contrast, when comparing threonine catabolism in rats and octopus (*Octopus vulgaris*), Akagi and Ohmori (2004) found that this amino acid is glucogenic in the cephalopod. Accordingly, (i) threonine is 25 times more abundant in octopus muscle (tentacles) than in rat liver, whereas glycine concentration is similar in both tissues; and (ii) D-lactate is formed 380 times faster in octopus tentacles than in rat muscles, according to Fujisawa et al. (2005), from threonine and glycine by the threonine dehydrogenase- and threonine aldolase-catalyzed reactions, in the presence of an amine oxidase activity for converting AA to MG and ultimately to D-lactate. In an attempt to clarify the metabolic role of D-lactate produced from MG, Fujisawa et al. (2005) compared the levels of D-lactate, pyruvate and MG in tissues of normal and starved *Octopus ocellatus*, as well as the activities involved in D-lactate metabolism, i.e., pyruvate kinase, glyoxalase I and II, and lactate dehydrogenases. The authors found that these enzymes were augmented in the mantle and tentacle of starved octopus, while



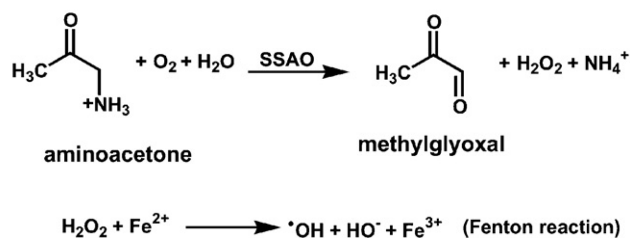
Scheme 6. Threonine metabolism.

the levels of D-lactate and related metabolites were lowered in these tissues. They also observed that D-lactate, pyruvate and MG were present at 320, 94 and 43 times higher concentrations in the tentacles of normally fed *O. ocellatus*, compared to rat skeletal muscle. Altogether these results confirm that D-lactate is actively used for energy production in the tentacles and mantle of starved animals. Interestingly, the D-lactate concentration in the tentacle of *O. ocellatus* was 17-fold higher than in *O. vulgaris*.

Boylan and Dekker (1981) were the first to purify threonine dehydrogenase, and later, Tressel et al. (1986) emonstrated that threonine dehydrogenase is physically associated with another enzyme, aminoacetone synthase (2-amino-3-oxobutyrate CoA-ligase), forming a "threonine cleavage complex" (TCC). The conversion of threonine into glycine by TCC is dependent on coenzyme-A (CoA), i.e., an abundance of CoA favors the conversion of threonine into glycine plus acetyl–CoA, while its absence favors spontaneous threonine decarboxylation to yield AA (Scheme 6). Working with isolated rat hepatocytes, House et al. (2001) recently found that threonine oxidation occurs mainly (~65%) via the glycine-independent threonine dehydratase pathway and is increased by glucagon. Since both NAD^+ and CoA are cofactors of threonine and glycine catabolism, the control of threonine degradation depends on the mitochondrial relations of NAD^+/NADH and CoA/acetyl–CoA (Davis and Austic, 1997). It is believed that in situations of ketoacidosis such as that of diabetes when acetyl–CoA levels are augmented, threonine is catabolized to AA, leading to its accumulation in the patients' tissues (Vander Jagt et al., 2001). One can anticipate that this also happens in individuals under Atkins diet (Astrup et al., 2004).

The disappearance of AA in rat liver proved to be dioxygen-dependent, as confirmed by a 90% decrease in the reaction rate when nitrogen was bubbled in the reaction mixture (Bird and Nunn, 1983). Ray and Ray (1983, 1987) described the formation of MG from AA, catalyzed by an amine oxidase present in the plasma and liver of ruminants. This enzyme was purified and studied with several amine substrates, but AA proved to be the most reactive of all (K_m=9 μM, for *Capra aegagrus hircus* hepatic enzyme), leading those researchers to believe that this enzyme is specific for MG formation in vivo from AA (Scheme 7). Later, the enzyme described by Ray and Ray was reported to be a semicarbazide sensitive amine oxidase (SSAO) whose substrates are primary amines, the most reactive of which are AA, methylamine (Boor et al., 1992) and benzylamine (non-endogenous) (Lyles, 1994).

SSAOs constitute a family of copper-dependent glycoproteins expressed in both prokaryotes and eukaryotes, in almost all mammalian tissues, with a molecular mass of almost 180,000 Da, and a carbonyl-containing cofactor of the 6-hydroxydopa quinone type or even pyridoxal or a pyrroloquinone (Lewinsohn, 1984). All these factors possess a carbonyl group sensitive to nucleophilic attack by "oxo-reagents" such as semicarbazide, arylamines, hydrazines and related compounds (Matyus et al., 2004). Because SSAOs are distributed in the vasculature and the plasma, their function is probably not only to produce MG and lactate from AA, but also to metabolize circulating amines, endogenous or not, that may exert pharmacological or deleterious activities (O'Sullivan et al., 2004; Matyus et al., 2004; Yu et al., 2003). Recently, upon isolating full-length SSAO cDNA from rat aorta and examining its mRNA expression in several rat tissues, Ochiai et al. (2005) found a 91% and 80% identity in the amino acid sequence of mouse and human SSAO, respectively.



Scheme 7. SSAO-catalyzed aminoacetone oxidation.

7. Methylglyoxal and diabetes mellitus

The discovery that diabetes is a metabolic state characterized, among other factors, by an increase in AA concentration and that the circulating SSAO is high in both type I diabetes mellitus (insulin-dependent, IDDM) and type II diabetes mellitus (insulin-independent, NIDDM) (Boomsma et al., 1999) has led several researchers to investigate the reaction mechanisms of this enzyme, to evaluate its activity in the tissues and plasma of patients, and to unveil its metabolic roles. The normal activity of SSAO in human plasma is *ca.* 350 mU/L; however, it is considerably higher in diabetic patients: 641 mU/L (type I) and 619 mU/L (type II) (Boomsma et al., 1999). Patients with congestive cardiac failure also have high levels of SSAO activity (590 mU/L), but there is little information about the cause of this increase (Boomsma et al., 1997).

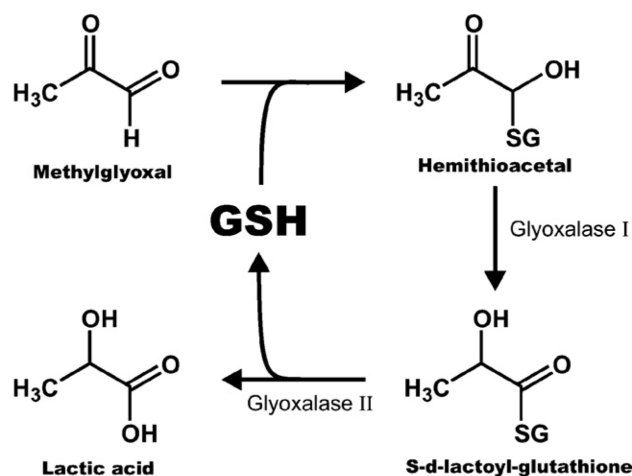
SSAO is distributed in various human tissues, the highest activity being found in the blood vessels (Lyles, 1994). It is also located in the retina and cerebral microvessels (Yu and Zuo, 1997), cartilages (Lyles and Mcdougall, 1989), uterus, urethra, deferent vessels (Lewinsohn, 1981), kidneys (McLellan et al., 1992) and in blood circulation (van Dijk and Boomsma, 1998). SSAO in mammals is predominantly connected to the plasmatic membrane of tissues, especially in smooth muscle cells (Chalmers and Lyles, 1995). It has been proven that vascular smooth muscle cell cultures secrete a soluble form of SSAO, indicating, as such, the possible origin of circulating SSAO (Hysmith and Boor, 1988). Although its metabolic function has not yet been unequivocally clarified (Lyles, 1994), it is believed to play an angiogenesis inhibitory role (Yu, 1998; Yu et al., 2003).

The SSAO-catalyzed oxidation of AA with the consequent formation of MG, hydrogen peroxide and oxyradicals, which are well-known cytotoxic species (Scheme 7), has been proposed as a potential source of ROS and a consequent promoter of the vascular dysfunctions observed in diabetic patients (Yu et al., 1994; Yu and Zuo, 1997; Yu, 1998; Yu et al., 2003). In recent years, MG has been associated with the redox imbalance found by various groups (Baynes, 1991; Yu, 1998; Kalapos, 1999a,b) in diabetes mellitus, but the mechanisms leading to this alteration have not yet been completely established. Neuropathy, nephropathy and retinopathy are some of the disorders related with accelerated atherosclerosis caused by diabetes in response to the action of MG on proteins, causing the formation of AGEs (Yu, 1998). According to Thornalley (1996), the estimated normal concentration of MG in human plasma lies in the range of 5 μM, but reaches values 5- to 6-fold higher in patients with type I diabetes and 2- to 3-fold higher in patients with type II diabetes. For comparison, using ESI/LC/MS analysis of a MG derivative with 2,3-diaminonaphthalene, Odani et al. (1999) found lower plasma MG values: 2 μM for type II diabetes, 1.5 μM for uremic patients, and 0.7 μM for normal individuals. More recently, Nemet et al. (2004) found even lower values of MG in human plasma samples upon treatment with 1,2-diamino-4,5-dimethoxybenzene: 708 nM for type II diabetic patients and 520 nM for the control group. ESI/LC/MS analysis of Sprague–Dawley rat tissue homogenates, after derivatization with 2,3-diaminobenzene, revealed that MG is about five-fold higher in the aorta (~11 nmol/g wet weight), than in the heart, liver, kidney or blood (Randell et al., 2005). These authors also state that this method may allow the evaluation of plasma MG in essential hypertension and type II diabetes.

According to Kalapos (1994, and references therein), MG can be formed in mammals by three metabolic routes that are initiated by triose phosphates, ketone bodies and threonine. The first two routes account for 90% of the amount of MG formed in the organism (Kalapos, 1992). In the latter, MG is produced by glycine and threonine, with the intermediacy of AA, as discussed earlier. Although this third route appears to be less important in the formation of MG in normal individuals (~10%), the fact that SSAO concentration is higher in tissues susceptible to damage caused in diabetes (Yu, 1998; Kalapos, 1994) supports the hypothesis that SSAO acts as a source of prooxidants (H_2O_2 and hydroxyl radical) and as a protein aggregator (MG) in these tissues.

Today it is accepted that the main route for MG detoxification includes the enzymatic system of the glyoxalases (Atkins and Thornalley, 1989). This system, described by Dakin and Dudley (1913) and Neuberg and Oertel (1914), contains two enzymes – glyoxalase I and glyoxalase II – proven to be able to transform α-oxoaldehydes into pyruvate or α-ketoglutarate from MG and DOVA, respectively. Both are metabolites involved in ATP production through the citric acid cycle.

There are still many open questions about the biological function of the glyoxalase systems present in the cytosol and organelles (mainly mitochondria) crucial to support life (Thornalley, 1990). It has been determined that glyoxalase II is the reaction rate-limiting enzyme, but in both types of diabetes, glyoxalase I activity is significantly high and glyoxalase II activity is very low (Thornalley, 1998). Because MG is present in high concentrations in diabetics, it is not surprising that *S*-D-lactoyl-glutathione accumulates (Scheme 8). Concomitant accumulation of MG and *S*-D-lactoyl-glutathione is a strong indication of a possible role of MG as a depletor of

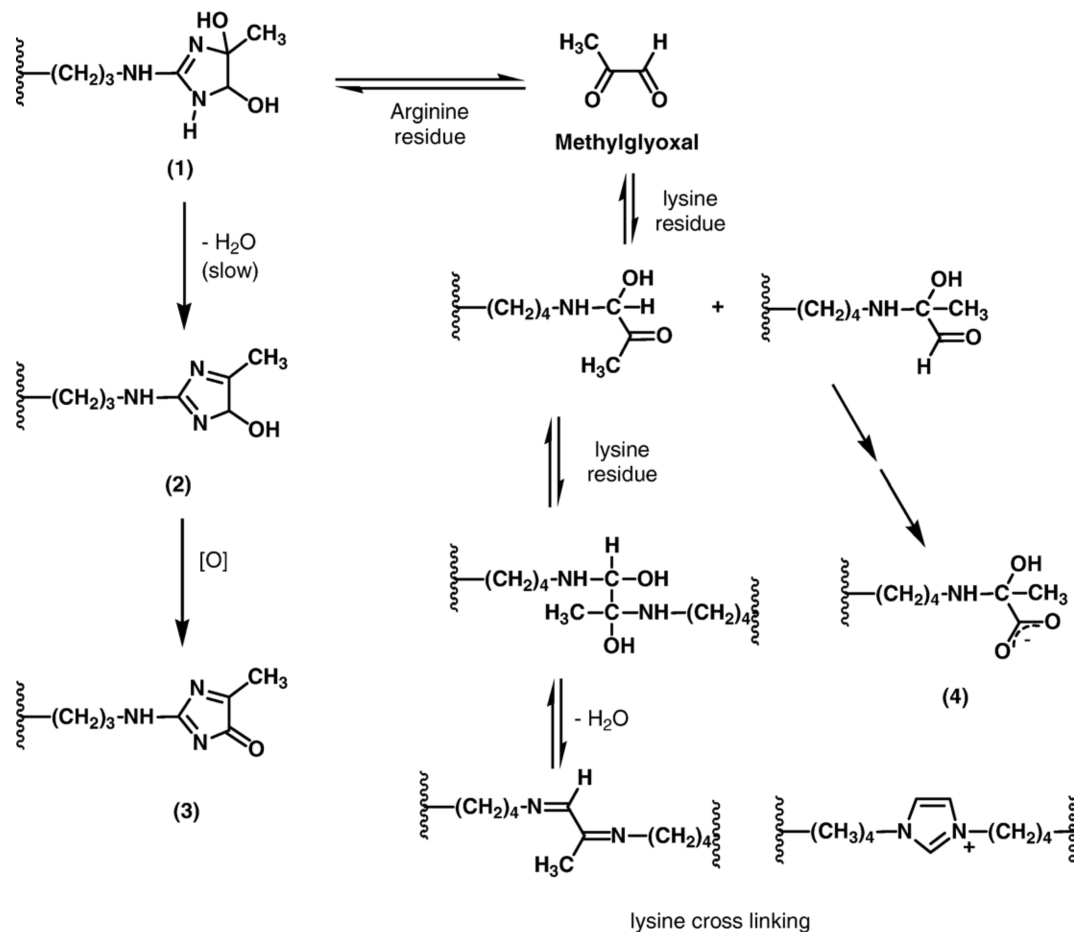


Scheme 8. Glyoxalase system.

reduced glutathione (GSH), important in the detoxification of H_2O_2 and lipid hydroperoxides. The final result is presumably the exacerbated production and deleterious activity of ROS in diabetes (Kalapos et al., 1993). Important reports in the literature, such as the concentration of MG in human crystalline lense being 20-fold higher than its plasma concentration (Haik et al., 1994) and very high in the kidneys and blood of diabetic patients (McLellan et al., 1992; Shipanova et al., 1997), reinforce the idea of a possible role of MG in the typical clinical manifestations of diabetes.

In addition to exhibiting prooxidant properties, MG has proved to be a potential endogenous mutagenic agent, as long foreseen by Szent-Györgyi (1941, 1968, 1973), by provoking multibase deletions (50%) and substituting base pairs (35%) in simian cells (Murata-Kamiya et al., 2000). The most important substitutions (transversions) observed *in vivo* were G:C→C:G and G:C→T:A (Murata-Kamiya et al., 2000). Doses of approximately 50 μM of MG are able to inhibit the growth of human leukemia 60 (HL60) cells, while doses of 360 μM of MG render cultures of these cells unviable (Ayoub et al., 1993). Another important observation is that MG inhibits the electron flow through complex I of the mitochondrial respiratory chain and inactivates glyceraldehyde-3-phosphate dehydrogenase of 'Ehrlich ascites' carcinoma cells and of leukocytes of leukemia patients, while exerting no effect on normal cells (Ray et al., 1994; Biswas et al., 1997).

We have already pointed out in this review that MG – an α-oxoaldehyde – can be involved in reactions with proteins called Maillard reactions (Monnier, 1989) through the initial formation of Schiff bases. It is known that MG can react with proteins to form fluorescent Maillard products. These reactions are initially reversible (Schiff condensation) and usually occur with arginine and lysine residues (forming glycosylamine derivatives) and also with cysteine residues (forming hemi-thioacetal derivatives) (Westwood and Thornalley, 1995a) (Scheme 9). This balance can be displaced through the formation of products (including pentosidines), arginine residues being the amino acid most susceptible to this type of modification (Westwood and Thornalley, 1995a). The resulting MG-modified proteins may attach to receptors at the cellular surface of monocytes and macrophages, where they undergo receptor mediated endocytosis and subsequent liposomal degradation (Westwood and Thornalley, 1995b; Westwood et al., 1997). Although glucose is the most abundant physiological sugar, its effective concentration in open chain aldehyde form is only 100 nM in the blood of normal individuals and 200–500 nM in diabetics. MG, on the other hand, is present in estimated concentrations of 10 μM in normal individuals and



Scheme 9. Reaction between methylglyoxal (MG) and arginine or lysine residues.

increased by 2- to 6-fold in diabetics, suggesting that MG may be as efficient as glucose *in vivo* in the modification of proteins (Kalapos, 1999a,b; Abordo et al., 1999; Thornalley, 1999; Chaplen et al., 1998; Shamsi et al., 1998; Bourajjaj et al., 2003; Ahmed et al., 2005). Innumerable papers have been published in the last few years describing *in vivo* and *in vitro* formation of MG adducts with protein arginine, cysteine, and lysine residues, resulting in the formation of AGEs (Westwood et al., 1997; Al-Abed et al., 1996; Odani et al., 1998; Lee et al., 1998; Ahmed et al., 1997). As stated above, under physiological conditions, MG reversible condensation reactions with protein arginine and cysteine residues are continued by irreversible stages leading ultimately to various adducts (9) such as N_{δ}-(4,5-dihydroxy-4-methyl-imidazolidine-2-yl)ornithine (**1**), N_{δ}-(5-hydro-5-methyl-imidazole-4-on-2-yl)ornithine (**2**) and N_{δ}-(5-methyl-imidazole-4-on-2-yl)ornithine (**3**) (Thornalley, 1999; Odani et al., 1998). The reaction of MG with lysine residues produces N_{δ}-(1-carboxyethyl)lysine (**4**) and cross-linking derivatives. Such adducts are similar to those deriving from the protein glycation process, except for the fact that α-oxoaldehydes preferentially modify arginine residues, whereas the main targets in protein glycosylation are lysine residues (Thornalley, 1999). MG adducts with thiol groups of plasma albumin were isolated from diabetic patients and showed to cause a loss of albumin antioxidant capacity (Faure et al., 2005).

Neurotoxic reactive dicarbonyl metabolites such as MG and glyoxal have been identified in the cerebrospinal fluid of Alzheimer's patients, the MG levels being two-fold higher than in healthy controls (Kuhla et al., 2005). Furthermore, Kovacic and Cooksy (2005) suggested a role of biacetyl metabolite in alcohol toxicity and addiction, and Faure et al. (2005) suggested that deleterious effects induced by carbonyl stress in diabetes could also originate from albumin Schiff reactions with dicarbonyls, with consequent loss of the protein plasma antioxidant capacity.

In addition to being an SSAO inhibitor, aminoguanidine can prevent the irreversible modification of human plasma proteins by MG (Lo et al., 1994). Lyles (1994) showed that aminoguanidine is an efficient inhibitor of AGEs formation from MG, leading to decreased formation of atherosclerotic plaques and the prevention of nephropathy in diabetic rats. By virtue of diminishing the progression of retinopathy, neuropathy and nephropathy in diabetic rats, preventing protein cross-linking and, thus, the formation of AGEs receptor recognition factors with concomitant dysfunction of endothelial cells, aminoguanidine has been considered a possible therapeutic agent for diabetes mellitus patients (Hammes et al., 1995; Cameron et al., 1992; Sugimoto and Yagihashi, 1997; Soulis-Liparota et al., 1991; Twigg et al., 2002; Takatori et al., 2004; Usta et al., 2004; Kopman et al., 2005). Recently, Webster et al. (2005) showed that MG is also toxic to human neuroblastoma cells in a dose-dependent manner above 150 μM (LD_{50} μM) and that pre-incubation of MG with carbonyl scavengers such as aminoguanidine, tenilsetam, and lipoic acid significantly reduced its toxicity. As MG-derived AGEs have been detected in age-related protein deposits in Alzheimer's and Parkinson's diseases, the authors suggested that the use of carbonyl chemical traps to reduce the neurotoxicity of MG and other reactive carbonyl compounds.

8. Threonine and *cri-du-chat*

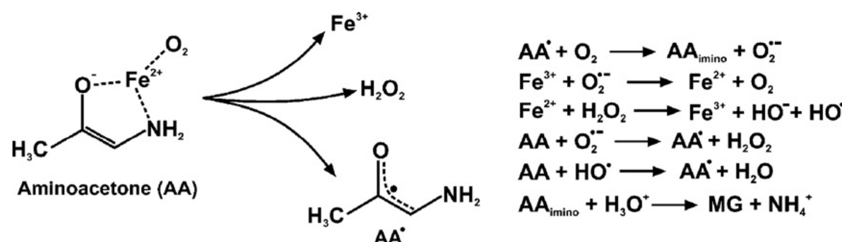
Cri-du-chat syndrome, described by Lejeune et al. (1963), is a chromosomal disorder that results from partial deletion, in the distal or interstitial part, of the short arm of chromosome 5 (5p−). This syndrome bears this name because its main characteristic, used as a diagnosis in newborns, is a monotonic cry similar to a cat's mew. Other characteristics of this syndrome are microcephaly, craniofacial deformations, hypertelorism and mental retardation (Niebuhr, 1978; Wilkins et al., 1980). Nevertheless, children with this syndrome are able to communicate their needs, interact socially with others, and have some degree of motility (Cornish and Pigram, 1996). Surveys conducted on *cri-du-chat* cases by Niebuhr (1978) in Denmark and by Higurashi et al. (1990) in Japan revealed an incidence of between 1:15,000 and 1:50,000. Perhaps because it is a rare syndrome, little is known about its biochemical or physiological features (Mainardi et al., 2000, and references therein).

In a study of amino acid metabolism in this syndrome, Kuhner et al. (1974) described an increase in the daily excretion of proline and threonine in *cri-du-chat* patients. More recently, Lejeune et al. (1990) observed a significant increase in the concentrations of asparagine and aspartate (Asx) in the plasma and urine of *cri-du-chat* patients, while histidine was increased only in the urine. The authors attribute the increase in Asx to a possible disorder in the metabolism of purines, while the increase in histidine could be connected to other deficiencies of the general metabolism of amino acids. Moreover, applying the strategy of "phenotype dissection" to the critical chromosome region for *cri-du-chat*, deletion of the gene encoding a ubiquitin-conjugated enzyme E2 type was found, suggesting that defective ubiquitin ligation may contribute to this genetic disorder (Swanson et al., 2001; Wu et al., 2005). Also relevant when discussing the molecular bases of the *cri-du-chat* syndrome is the correlation between deletions of δ-catenin, a neuron-specific catenin, and the severity of mental retardation, according to Israely et al. (2004).

Should the increase in circulating threonine in *cri-du-chat* patients be confirmed, a concomitant increase in AA concentration and the establishment of oxidative stress triggering the clinical manifestations of this disorder are to be expected.

9. Non-enzymatic oxidation of aminoacetone: a source of ROS in diabetes mellitus and *cri-du-chat*?

Dutra et al. (2001) demonstrated that AA rapidly reacts with molecular oxygen through a superoxide-propagated mechanism similar to that proposed for ALA (Scheme 4), however at a much faster rate than ALA, even in the presence of strong chelators, such as desferal, to immobilize eventual contaminant transition metals (Dutra et al., 2001). In the presence of catalytic iron, AA was found to consume dioxygen by another pathway, probably involving simultaneous transfer of two electrons (one from AA and the other from Fe(II)) to dioxygen, with direct

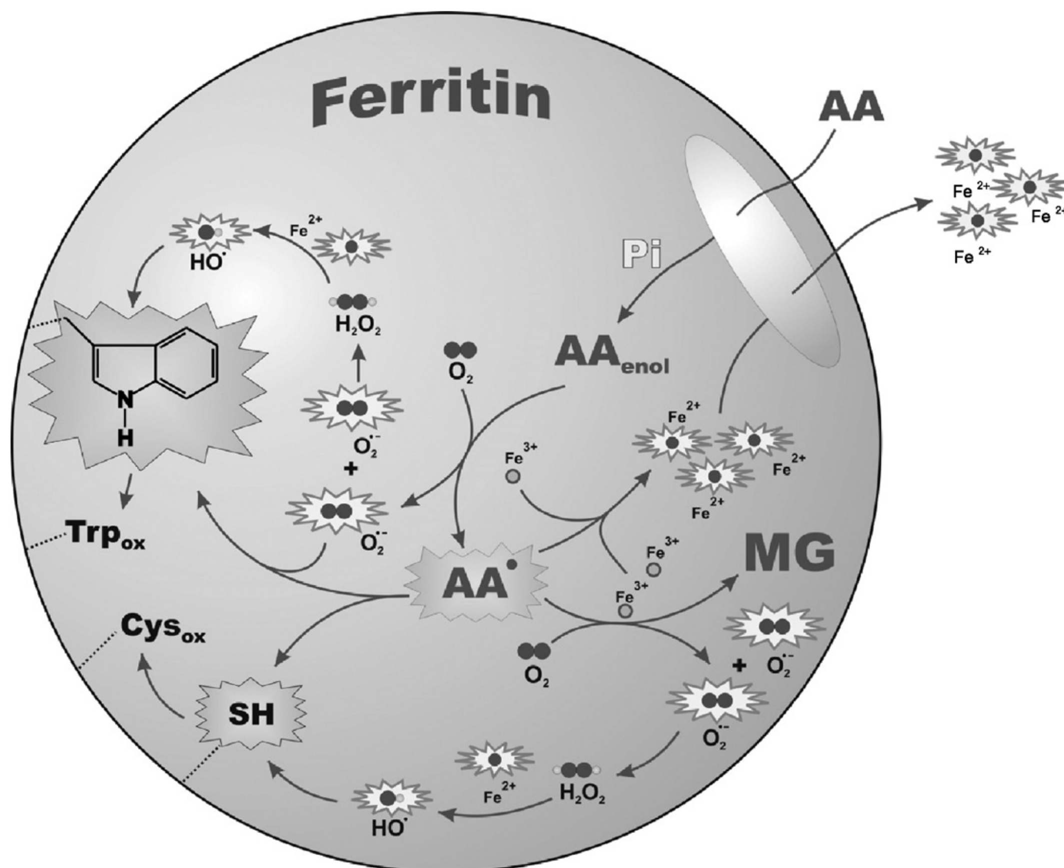


Scheme 10. Fe^{2+}-catalyzed aminoacetone (AA) aerobic oxidation.

formation of H_2O_2, enoyl AA˙ radical and Fe(III) (Scheme 10). This mechanism is supported by Wallace et al.'s early studies (1982) showing that nucleophile-catalyzed oxidation of oxyhemoglobin to metahemoglobin plus H_2O_2 involves previous binding of the electron donor to the external coordination sphere of iron(II), altering its reduction potential and favoring electron transfer to dioxygen.

Recently, Hiraku et al. (1999) reported copper-mediated oxidative damage promoted by AA on calf thymus DNA and on human DNA of cultured cells. The authors showed increments in the concentrations of 8-oxodG when DNA from calf thymus was incubated in the presence of AA and Cu(II). They also studied DNA cleavage and DNA ladder formation triggered by AA added to culture cells, which were inhibited by catalase and bathocupreine, suggesting that H_2O_2 and Cu(I) were involved. Product analyses led the authors to propose two different mechanisms for the Cu(II)-catalyzed oxidation of AA, both mediated by $O_2^{\cdot-}$: the major pathway in which AA is oxidized to MG and H_2O_2 and the minor one, whereby AA undergoes previous dimerization to 2,5-dimethyl-3,6-dihydropyrazine, which is oxidized by dioxygen to the corresponding pyrazine and H_2O_2. Polarographic and spectrophotometric studies performed with AA (0.5–10 mM)-containing buffered solutions, either air-equilibrated or nitrogen-purged, showed that AA direct oxidation dominates by far the dimerization reaction (Bechara and Sartori, unpublished).

The intermediacy of $O_2^{\cdot-}$ and OH˙ in ALA and AA aerobic oxidation (Schemes 4 and 10) and their effect on diverse biomolecular targets (Fig. 2) have been demonstrated by us *in vivo* and *in vivo* (Dutra et al., 2001, 2003, 2005). Chemical and



Scheme 11. Effects of aminoacetone on ferritin: release of Fe^{2+} and oxidation of Trp and Cys residues of their subunits, with loss of iron-storage ability and of ferroxidase activity.

enzymatic scavengers of ROS such as superoxide dismutase, catalase, semicarbazide, and spin traps were used to confirm the reaction mechanism. Preliminary studies showed that AA administered in millimolar amounts to insulin-producing cells (RINm5F), similar to the MG treatment reported by Sheader et al. (2001), led to cell death by apoptosis and necrosis (Bechara and Sartori, unpublished). As expected, cell mortality was hampered by added antioxidants such as catalase, superoxide dismutase, hydroxylamine and semicarbazide. Noteworth in this regard is the fact that many reported inhibitors of SSAO activity (e.g., azides, hydroxylamine, hydrazines, alkylphenylamines), used in millimolar amounts, are chemicals known to behave as superoxide radical scavengers, thus supporting the hypothesis that the SSAO-catalyzed reactions may also proceed via $O_2^{\cdot-}$, besides involving quinonimine intermediates (Matyus et al., 2004; Yu et al., 2003). No less interesting is the fact that transgenic mice expressing human SSAO showed an unusual pattern of elastin fibers in aorta, but did not display any kind of abnormality in the kidney and retina that would correspond to pathological changes (Gokturk et al., 2003). It is therefore tempting to propose that both SSAO-catalyzed and chemical oxidation of AA contribute to the biochemical damage and clinical manifestations of genetic disorders where AA accumulates.

We recently found that the rate of oxygen consumption by AA is augmented in the presence of horse spleen ferritin, resulting in the release of iron of this protein (Dutra et al., 2003). Ferritin is a protein directly related with iron storage in living organisms. Through its ferroxidase activity, ferritin is able to oxidize Fe(II) and uptake Fe(III) ion, thus playing an important role in the detoxification of this metal (Theil, 1987). In parallel, the ROS formed during the aerobic oxidation of AA were able, *in vitro*, to promote to ferritin and apoferritin sub-units: (i) modification of their electrophoretic behavior; (ii) oxidation of tryptophane and cysteine residues; (iii) loss of ferroxidasic activity; and (iv) decreased iron incorporation ability (Scheme 11). These effects on ferritin are relevant from a medical standpoint, for iron is known to trigger oxidative stress and tissue damage in both human and experimental diabetes (Niederau, 1999; Nickander et al., 1996; Young et al., 1995; Fernandez-Real et al., 2002). Aminoacetone has also been proven effective in provoking protein aggregation in ceruloplasmin (CP) (Dutra et al., 2005), a copper storage protein that has recently been implicated in iron homeostasis (Memisogullari and Bakan, 2004). AA-induced CP aggregation was accompanied by losses in its ferroxidase and aminoxidase activities, which are currently the focus of our studies directed at understanding the metabolic role of CP. According to Akaike (2000), CP catalyzes the unielectronic oxidation of nitric oxide (NO), producing intermediaries that are able to undergo nitrosative addition to thyol groups of proteins. These nitrosated proteins play an important role in signal transduction, genic transduction, apoptosis and oxidative stress.

Altogether, these data pose the question of whether threonine constitutes an important source of AA and ROS, underpinning the molecular bases of the pathophysiology of diabetes and *cri-du-chat*.

10. Hexosamine metabolism

Abundant data attest to the fact that the chronic accumulation of plasma glucose during diabetes mellitus may lead to both uncontrolled insulin secretion (type I diabetes) and insulin resistance (type II diabetes) (Anello et al., 1996; Eizirik et al., 1992; Purrello et al., 1989; Rossetti et al., 1990). As a result of chronic hyperglycemia, diabetics are subject to microvascular (responsible for blindness, neuropathies and kidney failure) and macrovascular (increased risk of myocardium infarction and amputations) complications (Ceriello, 2003). Macrovascular complications have been implicated as the leading cause of morbidity and mortality in diabetic patients and may set in well before diabetes is diagnosed (Gerich, 2005). The correlation between vascular damage and chronic hyperglycemia in diabetes mellitus was established upon the observation that endothelial cells exposed to hyperglycemia have an augmented flow of substrates both in glycolysis and in the tricarboxylic acid cycle (Brownlee, 2001, for a detailed review). Under this condition of substrate excess, there is an excessive increase in the mitochondrial oxidative processes, increase in the transmembrane potential and augmented production of $O_2^{\cdot-}$ by the electron transport chain (Hammes, 2003). These changes lead to a reduction in the activity of glyceraldehyde-phosphate dehydrogenase, thus accumulating the glycolytic intermediates fructose-6-phosphate (F6P) and glyceraldehyde-3-phosphate. Accumulation of F6P is responsible for triggering the metabolic pathway of the hexosamines, whose key enzyme is glutamine-F6P amidotransferase, responsible for the transformation of F6P into glycosamine-6-phosphate, which, in turn, is converted into uridine diphosphate-*N*-acetyl-glucosamine (GlcNAc) (Hammes, 2003; Cheatham, 2004).

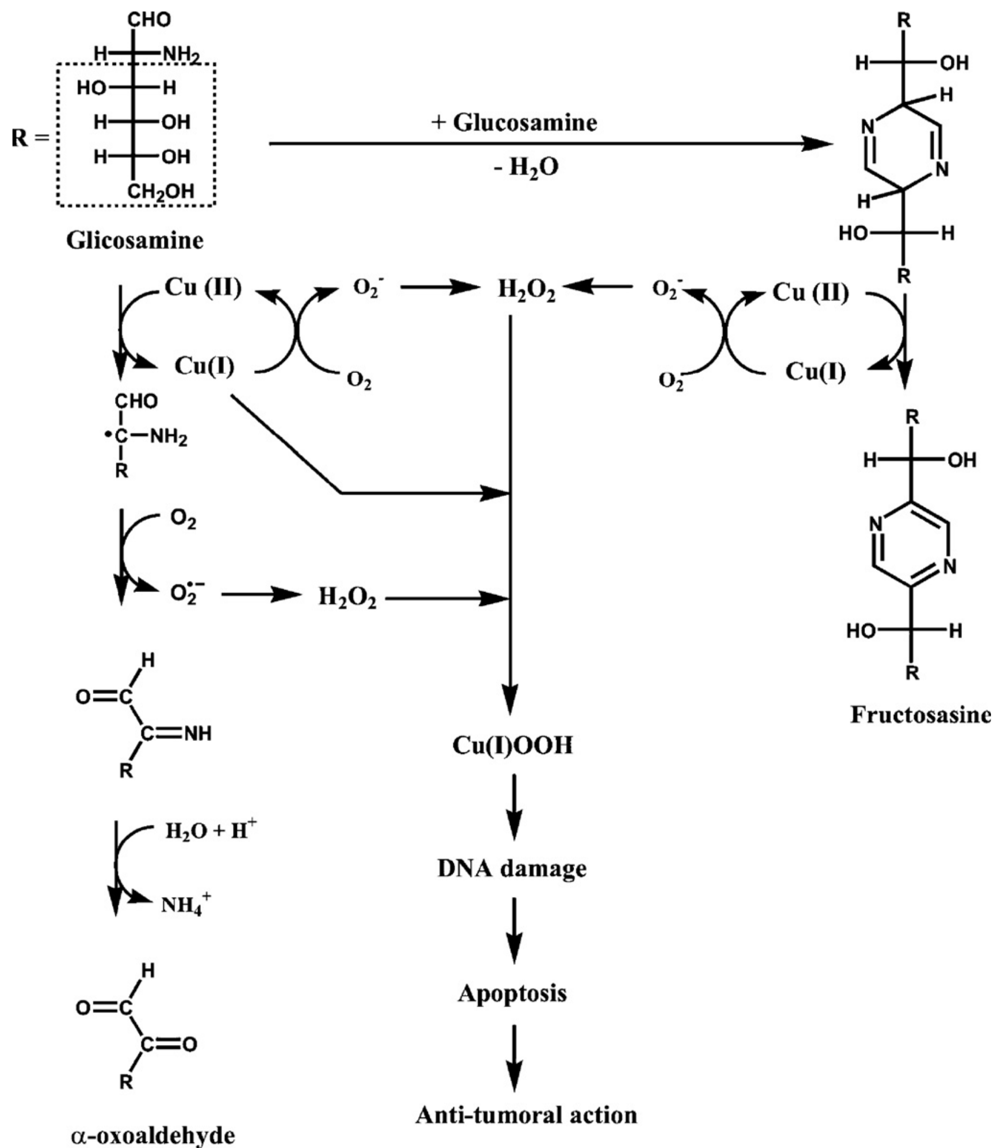
Various pieces of evidence point to the hexosamine pathway as a nutrient sensor system able to modulate cellular insulin sensitivity, since the intracellular levels of glucosamine and GlcNAc are associated with the availability of glucose, amino acids, fatty acids and nucleotides (Marshall et al., 1991; McClain, 2002; Lindsley and Rutter, 2004). Studies by Hazel et al., 2004 suggest that the high production of hexosamines in adipose mouse tissue suffices, by itself, to promote insulin resistance. Other studies demonstrate that hexosamines induce post-translational modification of transcription factors and of some cytosolic proteins, which could, in part, explain the molecular mechanism of insulin resistance (Brownlee, 2001). Moreover, glucosamine can activate the expression of obese (*ob*) gene in muscle cells and in adipocytes, exacerbating the excretion of leptin into plasma (Wang et al., 1998). Leptin is one of the circulating proteins synthesized and excreted in response to the increased storage of energy in adipose tissue, which are responsible for signaling nutritional satiation (Campfield et al., 1995).

In addition to a possible role in metabolic regulation, the hexosamines exert an inhibitory effect on tumor growth by inducing modifications in the biosynthesis of proteins, RNA and DNA in tumoral cells (Bekesi et al., 1969; Raisys and Winzler, 1971). According to Hiraku and Kawanishi (1999), a possible explanation for the antitumoral action of hexosamines is the induction of apoptosis by ROS formed during their aerobic

oxidation. These researchers demonstrated that hexosamines are subject to undergoing copper-catalyzed aerobic oxidation and promoting *in vitro* damage to calf thymus DNA. In this study, manosamine, fructosamine and glucosamine were shown to produce α-dicarbonylic compounds and ROS as oxidation products through two possible mechanisms (Scheme 12). The principal pathway involves the aerobic oxidation of hexosamine mediated by Cu(II), with the formation of a carbon-centered radical and Cu(I). This radical then reacts with oxygen to form $O_2^{\cdot-}$, whose dismutation generates H_2O_2, ultimately forming an α-dicarbonyl product and NH_4^+. The second pathway, less important and observable only in hexosamine concentrations higher than 1 mM, involves the condensation of two hexosamine molecules, forming a derivative of dihydropyrazine. This compound, in turn, presumably undergoes Cu(II)-catalyzed oxidation with the generation of $O_2^{\cdot-}$, H_2O_2 and a fructosazine derivative. In both routes, H_2O_2 reacts with Cu(I), forming a metal complex called copper peroxide (I) (Cu(I)OOH), with a hydroxylating activity similar to that of $HO^{\cdot}$ generated in the Fenton reaction (Oikawa and Kawanishi, 1998). The Cu(I)OOH complex can be formed in the DNA's surroundings, causing important structural modifications and subsequent triggering of apoptotic processes.

11. Final remarks

Several aspects of the biochemistry of endogenous α-aminoketones in humans, rats and other animals (quail, octopus, pig, etc) and their possible implications in redox inbalance were discussed here. Emphasis was placed on two α-aminoketones – ALA and AA – which have the potential to act *in vivo* as prooxidants in porphyrias, lead poisoning, tyrosinosis, diabetes, and *cri-du-chat* syndrome. Our hypothesis that ALA behaves as a source of deleterious ROS in intermittent acute porphyria, lead poisoning, hereditary tyrosinemia, and ALA-based photodynamic therapy seems to be widely accepted today



Scheme 12. Mechanism of glucosamine antitumoral action (Hiraku and Kawanishi, 1999).

by several investigators, among them Peng et al., 1997 (photodynamic therapy), Meyer et al., 1998 (neurological manifestations in IAP), Cadet et al., 1999a,b (metal-catalyzed DNA damage), Fuchs et al., 2000 (photodynamic therapy), Ryter and Tyrrel, 2000 (metabolism of the heme group as a source of prooxidants) and Gurer and Ercal, 2000 (antioxidants in the treatment of lead contamination).

Although AA proved *in vitro* to be a more effective prooxidant than ALA on ferritin, ceruloplasmin and isolated mitochondria, nothing is yet known about its putative prooxidant behavior *in vivo*. This is certainly a very challenging task, for AA reacts rapidly with molecular oxygen and its product, MG, forms Schiff adducts with proteins, making it difficult to determine the physiological and pathological concentrations of AA in plasma and tissues in order to establish correlations with the clinical manifestations of the diseases in which it accumulates. Hence the need to first unveil the mechanisms of AA reactions with several biomolecules, cell structures, and tissues, in both the presence and the absence of SSAO, to better understand its role in the oxidative stress and the deficit of mitochondrial respiratory function observed in diabetic patients.

Acknowledgements

The authors gratefully acknowledge the support of the CNPq-Conselho Nacional de Desenvolvimento Científico e Tecnológico, the FAPESP-Fundação de Amparo à Pesquisa do Estado de São Paulo, the PRONEX-Programa de Apoio a Núcleos de Excelência (Brazil), and the Programa do Milênio-Redoxoma, CNPq/MCT.

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