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REVIEW

The Pharmacology of the Antioxidant Lipoic Acid

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ABSTRACT. 1. Lipoic acid is an example of an existing drug whose therapeutic effect has been related to its antioxidant activity.

2. Antioxidant activity is a relative concept: it depends on the kind of oxidative stress and the kind of oxidizable substrate (e.g., DNA, lipid, protein).

3. *In vitro*, the final antioxidant activity of lipoic acid is determined by its concentration and by its antioxidant properties. Four antioxidant properties of lipoic acid have been studied: its metal chelating capacity, its ability to scavenge reactive oxygen species (ROS), its ability to regenerate endogenous antioxidants and its ability to repair oxidative damage.

4. Dihydrolipoic acid (DHLA), formed by reduction of lipoic acid, has more antioxidant properties than does lipoic acid. Both DHLA and lipoic acid have metal-chelating capacity and scavenge ROS, whereas only DHLA is able to regenerate endogenous antioxidants and to repair oxidative damage.

5. As a metal chelator, lipoic acid was shown to provide antioxidant activity by chelating Fe^{2+} and Cu^{2+}; DHLA can do so by chelating Cd^{2+}.

6. As scavengers of ROS, lipoic acid and DHLA display antioxidant activity in most experiments, whereas, in particular cases, pro-oxidant activity has been observed. However, lipoic acid can act as an antioxidant against the pro-oxidant activity produced by DHLA.

7. DHLA has the capacity to regenerate the endogenous antioxidants vitamin E, vitamin C and glutathione.

8. DHLA can provide peptide methionine sulfoxide reductase with reducing equivalents. This enhances the repair of oxidatively damaged proteins such as α-1 antiprotease.

9. Through the lipoamide dehydrogenase–dependent reduction of lipoic acid, the cell can draw on its NADH pool for antioxidant activity additionally to its NADPH pool, which is usually consumed during oxidative stress.

10. Within drug-related antioxidant pharmacology, lipoic acid is a model compound that enhances understanding of the mode of action of antioxidants in drug therapy. GEN PHARMAC 29;3:315–331, 1997. **© 1997 Elsevier Science Inc.**

KEY WORDS. Antioxidant pharmacology, dihydrolipoic acid, glutathione, lipoic acid, metabolism, metal chelation, peptide methionine sulfoxide reductase, reactive oxygen species, scavenging, vitamin C, vitamin E

ANTIOXIDANT PHARMACOLOGY

Within pharmacology, a new area of research concentrates on the pharmacology of antioxidants. Whereas at the beginning of the 20th century, P. Ehrlich noticed that most drugs act through binding to DNA or proteins (receptors, enzymes, carrier molecules, ion channels), more recently, other mechanisms by which a drug can affect the function of a living system are being recognized. By acting as an antioxidant, a drug may also affect the functioning of an organism. B. Halliwell, a leading scientist in the field of antioxidant research, formulated the definition of an antioxidant as: *any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate* (Halliwell, 1995). With this definition, antioxidants are of interest to anyone who wants to preserve goods: radiation chemists, food scientists and museum curators. The human body also needs to be preserved well. Antioxidants such as vitamin C, vitamin E and glutathione protect tissue from oxidative damage. Of course, the statement that living organisms contain natural preservatives is far too popularized. In living systems, a wide range of oxidative processes take place, delicately tuned by specific antioxidants. In some situations, the balance between the oxidative and the antioxidative processes tips in favor of the oxidative processes. This results in oxidative stress and may finally lead to pathology. Antioxidant pharmacology investigates possibilities of therapeutic intervention in oxidative processes (Bast, 1994). Basically, this is done through two routes (Fig. 1). The first route concentrates on existing or potential drugs. Compounds are screened for antioxidant activity, and the contribution to a pharmacological effect is evaluated. The second route concentrates on the pathology. Pathological processes are investigated, and the involvement of oxidative stress is considered. An example of an existing drug of which the therapeutic effect is related to its antioxidant activity is lipoic acid (Bast *et al.*, 1988). Ideally, both research routes converge and result in an effective and

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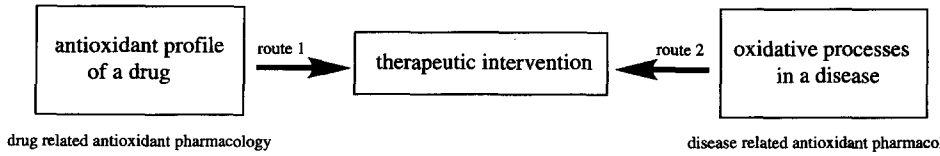


FIGURE 1. Antioxidant pharmacology investigates possibilities for therapeutic intervention in oxidative processes.

therapeutic intervention. In this review, we discuss the first route: research related to the antioxidant activity of the drug lipoic acid.

LIPOIC ACID

Lipoic acid (chemical name: 1,2-dithiolane-3-pentanoic acid, Fig. 2) is present in all kinds of prokaryotic and eukaryotic cells. In human beings, it is part of several 2-oxo acid dehydrogenases that take part in energy formation. Linked to lysine residues of the 2-oxo acid dehydrogenase multienzyme complexes (Fujiwara *et al.*, 1995; Morris *et al.*, 1995), lipoic acid acts as a cofactor. It binds acyl groups and transfers them from one part of the enzyme complex to another. In this process, lipoic acid is reduced to dihydrolipoic acid (DHLA), which is subsequently reoxidized by lipoamide dehydrogenase (Lip-DH) under the formation of NADH. Overall, lipoic acid and DHLA act as a redox couple, carrying electrons from the substrate of the dehydrogenase to NAD^+.

In 1966, German physicians started to administer lipoic acid to patients with liver cirrhosis, mushroom poisoning, heavy metal intoxication and diabetic polyneuropathy. Originally, the rationale for this treatment was the observation that patients with liver cirrhosis, diabetes mellitus and polyneuropathy had lower levels of lipoic acid (Kleemann *et al.*, 1989). It was assumed that supplementation with lipoic acid helped to overcome the shortage, thereby restoring the 2-oxo acid oxidation. Indeed, destruction of the cofactor function of lipoic acid may be involved in pathological processes. In arsenite intoxication, As^{3+} can form a complex with lipoic acid in the 2-oxo acid dehydrogenases, rendering it inactive. In certain types of liver disease, autoantibodies that recognize lipoylated subunits in the multienzymes have been demonstrated in patients' sera (Gut *et al.*, 1995; Tuaillon *et al.*, 1992). Also, oxidative stress near the dehydrogenase complex can lead to oxidative destruction (Gutierrez Correa and Stoppani, 1996), thereby adversely affecting the functioning of lipoic acid as a cofactor.

Lipoic acid is bound to proteins and, consequently, free lipoic acid has not been detected in human beings (Hermann *et al.*, 1996). However, after therapeutic application, free lipoic acid can be found in the circulation (Teichert and Preiß, 1995). It is likely that the therapeutic effects originate from free, unbound lipoic acid. In heavy metal intoxications, free lipoic acid and its metabolites are

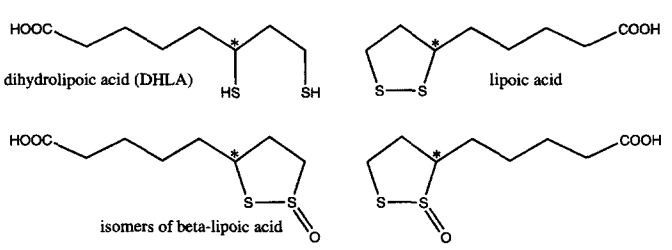


FIGURE 2. The chemical structures of dihydrolipoic acid (DHLA), lipoic acid and the isomers of β-lipoic acid. The structures contain a chiral center (*). The R-configuration is naturally occurring.

able to trap the metals in the circulation, thereby preventing the damage caused by the metal and (sometimes) improving its secretion. In diabetic polyneuropathy, free lipoic acid may enter nerve tissue and prevent glucose-related oxidative damage.

Especially in diabetic polyneuropathy, approaching antioxidant pharmacology via the pathology- and drug-related routes may lead to promising possibilities for therapeutic intervention. It has been shown that oxidative stress is part of the pathological process of diabetes. It has been suggested that oxidative stress has a role in the causation of noninsulin-dependent diabetes (Salonen *et al.*, 1995). Wolff *et al.* (1991) demonstrated that high glucose levels produce oxidative stress. In streptozotocin diabetic rats, antioxidants and lipoic acid delayed the onset of polyneuropathy (Cameron *et al.*, 1993; Nagamatsu *et al.*, 1995; Vertommen *et al.*, 1994). In clinical trials, lipoic acid did not improve polyneuropathy over a relatively short period of 3 weeks. The benefit of lipoic acid was found in reducing neuropathic complaints such as pain and paresthesia (Ziegler *et al.*, 1995). Although the exact role of oxidative stress in human diabetics is still under investigation (route 2), the obtained results warrant a further investigation of the antioxidant role of lipoic acid in oxidative stress (route 1).

OXIDATIVE STRESS

The functioning of living systems depends on energy. Aerobic organisms derive their energy from the oxidation of fuel molecules such as glucose and fatty acids. In oxidation, electrons are removed from these molecules and subsequently transferred in a chain of reactions to other molecules until they finally reach their ultimate electron acceptor: O_2. Often, oxidative stress originates from improper control of this reduction of O_2.

As already noted, an important molecule in oxidative stress is molecular oxygen, O_2 (Halliwell and Gutteridge, 1989). In its ground state, O_2 has two unpaired electrons in its antibonding π^* molecular orbital (Fig. 3). More reactive forms of oxygen are the singlet oxygens: $^1\Delta gO_2$ and $^1\Sigma g^+O_2$, 22.4 and 37.5 kcal above the ground state of O_2, respectively. If a single electron is added to O_2, it enters one of the π^* antibonding orbitals. The product is the superoxide radical $O_2^{\bullet -}$, with only one unpaired electron. Consequently, addition of one more electron will give a nonradical species, the peroxide ion O_2^{2-}, which forms, in the presence of protons, H_2O_2. Because the extra electrons in $O_2^{\bullet -}$ and O_2^{2-} are entering antibonding orbitals, the strength of the oxygen–oxygen bond decreases. The addition of two more electrons to O_2^{2-} eliminates the bond entirely because the electrons go into σ^*2p orbitals, thus producing $2O^{2-}$ species. Altogether, in physiology, the two-electron reduction product of O_2 is H_2O_2, and the four-electron product is H_2O.

Some transition metals can participate in the chain of O_2 reduction. Oxidized metals, such as Fe^{3+} and Cu^{2+}, catalyze electron transfer from one oxygen species to another. For example, the dismutation of $O_2^{\bullet -}$ is accelerated in the presence of Fe^{3+}:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{3+} + O_2 \qquad (1)$$

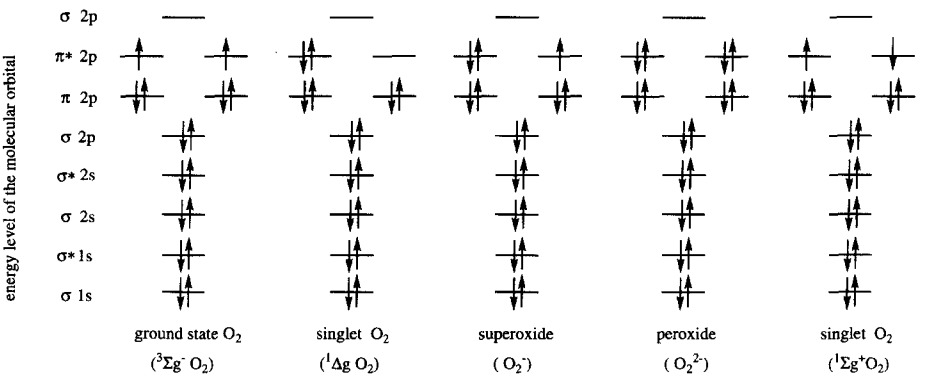


FIGURE 3. Molecular-orbital energy pattern for the diatomic oxygen molecule.

$$Fe^{2+} + O_2^{\bullet -} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (2)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (3)$$

Reduced metal ions, such Fe^{2+} and Cu^+, can donate one electron and thus start a whole sequence of reactions. An example is the Fenton reaction in which the very reactive hydroxyl radical (•OH) is formed:

$$Fe^{2+} + H_2O_2 \rightarrow \text{intermediate complex} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad (4)$$

Traces of Fe^{3+} might be able to react further with H_2O_2, although this is very slow at physiological pH:

$$Fe^{3+} + H_2O_2 \rightarrow \text{intermediate complex} \rightarrow Fe^{2+} + O_2^{\bullet -} + 2H^+ \quad (5)$$

Even more reactions are possible:

$$^{\bullet}OH + H_2O_2 \rightarrow H_2O + H^+ + O_2^{\bullet -} \quad (6)$$

$$O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad (7)$$

$$^{\bullet}OH + Fe^{2+} \rightarrow Fe^{3+} + OH^- \quad (8)$$

The overall sum of these reactions is the iron-catalyzed decomposition of hydrogen peroxide:

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad (9)$$

In aerobic organisms, almost all steps in the reduction of O_2 are catalyzed by metals. Mostly, the reactivity of the metals is controlled by incorporating the metal in an enzyme. Examples of such enzymes are superoxide dismutase (SOD), catalase and cytochromes. Dissociated from their protein environments, metals take part in other, often unwanted, secondary reactions (e.g., in the Haber-Weiss reaction). The protein environment of SOD prevents the metal-catalyzed reaction between $O_2^{\bullet -}$ and H_2O_2, but unbound iron or copper induces hydroxyl radical formation:

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \quad (10)$$

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \quad (11)$$

$$O_2^{\bullet -} + H_2O_2 \rightarrow {}^{\bullet}OH + O_2 + OH^- \quad (12)$$

In the absence of iron, the reaction rate of the Haber-Weiss reaction is virtually zero. This illustrates the extreme importance of iron and copper in the formation of •OH.

The species H_2O_2 and •OH, as well as other oxygen-derived species such as $^1\Delta gO_2$ and HOCl, can damage biomolecules such as DNA, proteins and lipids. They are generally referred to as reactive oxygen species (ROS). Many ROS were ultimately derived from $O_2^{\bullet -}$. The proposal that $O_2^{\bullet -}$ is the major factor in oxygen toxicity is known as the superoxide theory of oxygen toxicity. Superoxide anions can be formed *in vivo* through several routes. First, some biologically important molecules and certain xenobiotics can oxidize in the presence of O_2 to yield $O_2^{\bullet -}$: they include glyceraldehyde, reduced forms of riboflavin, hemoglobin, adrenaline, tetrahydropteridines, thiols, paraquat and doxorubicin. Second, the electron transport chains of mitochondria and the endoplasmatic reticulum can "leak" electrons to O_2. In mitochondria, cytochrome oxidase, NADH-coenzyme Q reductase and reduced forms of coenzyme Q itself are sites of electron leakage. In the endoplasmatic reticulum, improper reactions of the cytochrome P-450 enzymes can "leak" electrons to O_2. A third source of $O_2^{\bullet -}$ are the phagocytic cells. These cells contain a membrane-bound enzyme, NADPH oxidase, which catalyzes the formation of $O_2^{\bullet -}$ according to the following stoichiometry:

$$NADPH + 2O_2 \rightarrow 2O_2^{\bullet -} + H^+ + NADP^+ \quad (13)$$

The toxic effect of ROS usefully contributes to microbicidal activity of the phagocytic cells. Although all ROS originate from O_2 reduction, not all ROS are derived from $O_2^{\bullet -}$. For example, NO• is formed by NO synthase in an oxidative process resembling a P-450 reaction (Boucher *et al.*, 1992). In this process, reduced oxygen is incorporated into an N^{ω}-OH-*L*-arginine intermediate (Nathan, 1992).

When compounds with one or more unpaired electrons are small and freely diffusible, they are referred to as "free radicals." In this view, ROS—just like O_2, $O_2^{\bullet -}$, •OH and metals ions with unpaired electrons in the *d* orbitals—are considered free radicals. Apart from some exceptions, most reactions in chemistry can be described as polar or radical reactions. In polar reactions, bonds are made when an electron-rich reagent donates a pair of electrons to the electron-poor reagent. On the other hand, in radical reactions, each reactant donates only one electron to the new bond. Biologists have been assuming for years that free radicals do not exist in biological systems. However, the observation that radical reactions also take place in biology has aroused a new trend: "free radical research." Although this terminology is logical on a historical basis, chemically it is awkward. It suggests that it is useful to study "free radicals" and, for example, "free anions" separately. Nowadays, scientists are aware of this strange terminology and have started to acknowledge the total chain of oxidative processes, regardless of their radical or polar character.

ANTIOXIDANTS

To control oxidative processes, biological systems have been equipped with several antioxidant mechanisms. Antioxidant enzymes such as SOD, catalase and peroxidases are concerned with the removal of $O_2^{\bullet -}$ and H_2O_2. Metal ions can be sequestrated to inhibit uncontrolled dismutation of $O_2^{\bullet -}$, the Fenton reaction and the Haber-Weiss reaction. ROS can be scavenged in an aqueous environment by small molecules such as vitamin C, glutathione and uric acid. In a lipid environment, vitamin E scavenges ROS-derived radicals and protects the membranes. When, in spite of all this, the antioxidant system has failed, damage to endogenous molecules may be repaired. Organisms contain for this purpose DNA repair systems, protein turnover mechanisms and peptide methionine sulfoxide reductase (PMSR).

As stated before, drugs also have antioxidant activity. Generally, the antioxidant properties of a drug are determined by their chemical and physical properties. The combination of these properties and the concentration determine its final antioxidant activity. To be effective, the drug itself and its relevant metabolites should become available to the tissue that is prone to oxidative stress. Within antioxidant pharmacology, the absorption, distribution, metabolism and excretion of a drug are studied. The antioxidant properties of the drug and its metabolites are examined. For the evaluation of the antioxidant properties of lipoic acid, four aspects have been distinguished:

1. The metal-chelating capacity
2. The ROS-scavenging capacity
3. The capacity to regenerate endogenous antioxidants
4. The role in repair systems

The four antioxidant properties can be studied as pure chemical reactions. The corresponding antioxidant activity is determined in relation to prevention of oxidative damage. The antioxidant activity is assessed in *vitro* or *in vivo*. For lipoic acid, the experiments that have been performed to test its bioavailability, metabolism and antioxidant activity are reviewed in the next section.

THE BIOAVAILABILITY OF LIPOIC ACID

Our daily diet contains lipoic acid. Especially, food derived from tissue with a high metabolic activity has a high lipoic acid content (Herbert and Guest, 1975; Mattulat and Baltes, 1992). Meat from a metabolic organ such as pig heart has a lipoic acid content of 1.1–1.6 mg/kg, whereas calf muscle contains only 0.07–0.15 mg/kg (Mattulat and Baltes, 1992). This indicates that most lipoic acid in the diet originates from the multienzyme complexes. Proteolytic enzymes do not effectively cleave the peptide bound between lipoic acid and lysine. Therefore, it has been suggested that, after digestion, lipoic acid is absorbed as lipoyllysine (Mattulat, 1992). In addition, lipoic acid can be obtained by *de novo* biosynthesis from fatty acids and cysteine (Carreau, 1979; Spoto *et al.*, 1982). Indications were found that lipoic acid is synthesized in a protein-bound form (Spoto *et al.*, 1982). From food or biosynthesis, only minor amounts of free lipoic acid will enter the circulation. After oral application, free lipoic acid is obtained in relatively high amounts. The therapeutic dose exceeds the dietary intake by far. Doses have been administered up to 10 mg/kg IV to rat (Stroman *et al.*, 1989) and to human beings up to 1,200 mg IV (Ziegler *et al.*, 1995), which is equal to the lipoic acid content of *circa* 1,000 kg of pig heart.

After administration of lipoic acid, its concentration can be determined in the blood plasma (Teichert and Preiß, 1995) and in the tissue (Maitra *et al.*, 1996). Originally, all analytical methods for the determination of lipoic acid contained a hydrolysis step to liberate lipoic acid bound to the lysine residue. Because free lipoic acid is thought to be the most important therapeutic form, nowadays free lipoic acid concentrations are evaluated (Biewenga *et al.*, 1996c; Handelman *et al.*, 1994; Hermann *et al.*, 1996). Figure 4 shows that concentrations up to 1,154 ng/ml of free lipoic acid appear in the blood plasma after oral application of 1 g of *R*-lipoic acid to a healthy male volunteer. Hermann *et al.* (1996) extensively studied the pharmacokinetics and bioavailability of racemic lipoic acid in different formulations. The half life in plasma is approximately 30 min (as is also seen in Fig. 4). The mean total plasma clearance was in the same range as the plasma flow of the liver (about 11–17 ml/(min kg). The liver presumably eliminates lipoic acid. The absolute bioavailability (F_{abs}) was calculated to be between 20% and 38%, depending on the isomer and formulation.

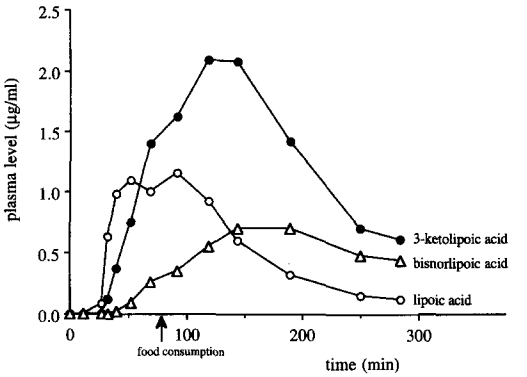


FIGURE 4. The time course of the plasma concentration of lipoic acid (open circles), 3-ketolipoic acid (closed circles) and bisnorlipoic acid (triangles) after oral administration of 1 g R-lipoic acid to a healthy volunteer who had fasted overnight. A meal high in fat content was consumed 85 min after oral intake of R-lipoic acid. The compounds were analyzed by high-performance liquid chromatography coupled to electrochemical detection.

A relation between bioavailability and a pharmacological effect caused by antioxidant activity has not yet been established. A relation between lethality and bioavailability seems to exist. Bioavailability explains the difference in LD_{50} after oral and other routes of application (Table 1). From extrapolation of these data, human beings would tolerate several grams of lipoic acid. Indeed, no serious side effects have been reported even after administration of about 1 g of lipoic acid. Injection (i.v.) of 1,200 mg of lipoic acid induced adverse reactions such as nausea or vomiting in 28 of 86 diabetic patients (Ziegler *et al.*, 1995). After i.m. injection of 40 mg, a burning pain on the place of injection was reported (von Schreiber, 1961).

THE METABOLISM OF LIPOIC ACID

Reduction

Lipoic acid can be reduced to the dithiol DHLA (Fig. 2). Because this reduced form greatly contributes to the antioxidant activity of

TABLE 1. The dose at which 50% of the animals die after application of lipoic acid

Application	LD_{50} rat	LD_{50} mouse
p.o.	1,130 mg/kg	502 mg/kg
i.p.	200 mg/kg	160 mg/kg
s.c.	230 mg/kg	200 mg/kg
i.v.	180 mg/kg	210 mg/kg

Adapted from Lewis and Sweet, 1985.

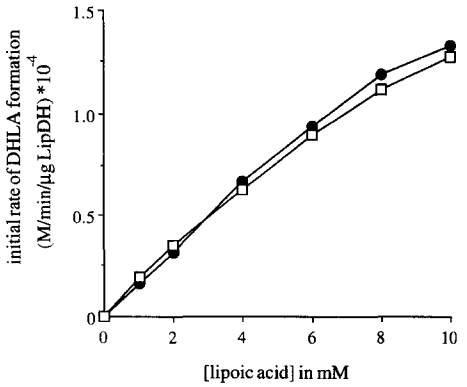
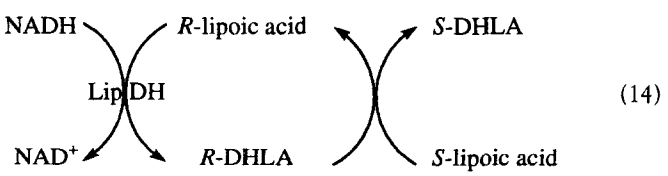


FIGURE 5. The initial rate of DHLA formation from racemic lipoic (squares) acid and *R*-lipoic acid (circles) by LipDH. For comparison, the observed rate of racemic lipoic acid was multiplied by two. Within the first 60 sec, DHLA formation was monitored at 500 nm using the thiol reagent dithionitrobenzoic acid in the presence of 1.0 mM NADH. The reaction was started by addition of LipDH (5 µg/ml).

lipoic acid *in vivo*, mechanisms of reduction have been studied. It is known that disulfides can be reduced by other thiols. Under normal conditions, the thiol glutathione (reduced, GSH) is present abundantly. In principle, GSH is able to reduce the intramolecular disulfide of lipoic acid. However, this reaction proceeds so slowly that substantial reduction of lipoic acid by GSH was never observed (Biewenga *et al.*, 1996b). More important is the enzymatic reduction of lipoic acid. In mitochondria, LipDH can also perform the reverse reaction of the 2-oxo acid dehydrogenases. It catalyzes the reduction of free lipoic acid at the expense of NADH. In the cytosol, GSH reductase catalyzes the reduction at the expense of NADPH. Remarkably, both enzymes have opposite stereospecificity. Mammalian GSH reductase catalyzes the reduction of *S*-lipoic acid about twice as fast as the *R*-lipoic acid reduction (Pick *et al.*, 1995). Mammalian LipDH reduces the other enantiomer, *R*-lipoic acid, approximately 28 times as fast as the *S*-enantiomer (Biewenga *et al.*, 1996a; Löffelhardt *et al.*, 1995).

Therapeutically, lipoic acid is administered as a racemic mixture. Originally, it was thought that *S*-lipoic acid, being a poor substrate, could occupy the active site of LipDH and inhibit the reduction of *R*-lipoic acid. Indeed, it was shown that *S*-lipoic acid can act as an inhibitor of LipDH (Biewenga *et al.*, 1996a; Löffelhardt *et al.*, 1995). However, at saturating NADH concentrations (above 0.1 mM), no decreased DHLA formation was observed for the racemic mixture (Fig. 5). Apparently, R-DHLA formation is not affected by *S*-lipoic acid. Therefore, *S*-lipoic acid should be regarded as an isomeric ballast in the LipDH-dependent reduction of racemic lipoic acid. However, the stereoselectivity of the reduction of racemic lipoic acid is not as strict as predicted. DHLA is a strong reductant. It is able to reduce disulfide groups, as well as the intramolecular disulfide in lipoic acid. After administration of racemic lipoic acid, *R*-DHLA and *S*-lipoic acid will coexist in the same cell compartment. The nonenzymatic reduction of one isomer by the reduced form of the other occurs.



(14)

For the reduction catalyzed by GSH reductase, S-DHLA may non-

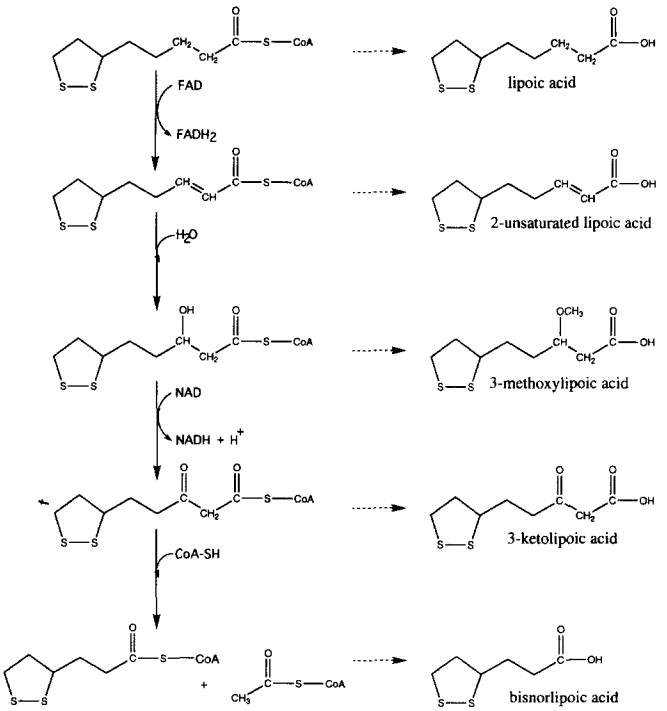


FIGURE 6. The chemical structure of the principal metabolites of lipoic acid that have been identified.

enzymatically reduce *R*-lipoic acid. This implies that the poorly reduced form should not be considered isomeric ballast. Overall, on a theoretical basis, it is expected that the reduction of a racemic mixture would be superior to that of both isomers administered apart. *In vivo*, detection of DHLA is hampered by its high reactivity. In human plasma, for example, DHLA added to plasma instantly reacts with plasma components (Biewenga *et al.*, 1996c; Teichert and Preiß, 1995). However, formation of DHLA was proved by using human erythrocytes (Constantinescu *et al.*, 1995) and rat eye lenses. By application of lipoic acid to newborn rats, the racemic form produced more DHLA in the lenses than did *R*-lipoic acid or *S*-lipoic acid alone. Coincidentally, racemic lipoic acid showed more protection against buthionine sulfoximine (BSO)-induced cataract than did the pure isomers (Maitra *et al.*, 1996).

β-oxidation

Another metabolic event of lipoic acid is the β-oxidation of its pentanoic acid side chain (Fig. 6). In the bacterium *Pseudomonas putida* LP, bisnorlipoic acid, tetranorlipoic acid and β-hydroxybisnorlipoic acid were identified as principal metabolites of β-oxidation (Furr *et al.*, 1978). In urine of rats, the same products plus a keto compound were detected (Spence and McCormick, 1976). In addition, CO_2 is a product of β-oxidation, produced by degradation of acetyl CoA (Fig. 6) in the citric acid cycle. Indeed, after the administration of [1,6-^{14}C]lipoic acid to rats, $^{14}CO_2$ was expired (Harrison and McCormick, 1974). Further support for β-oxidation has been provided by Harrison and McCormick (1974). Incubation of the [^{14}C]lipoic acid with mitochondrial preparations resulted in the production of $^{14}CO_2$, which could be decreased by unlabeled fatty acids.

In human beings, the metabolism was studied only recently. It was found that β-oxidation also occurs in human beings. In human plasma, bisnorlipoic acid was detected (Fig. 4). The maximum concentration of bisnorlipoic acid (704 ng/ml) was observed 189 min after oral administration of 1 g of *R*-lipoic acid to a male volunteer

(Biewenga *et al.*, 1996c). In urine, the main metabolite was S^4,S^6-dimethylbisnorlipoic acid (Locher *et al.*, 1995), indicating that β-oxidation products are further metabolized before they are excreted in urine.

In conclusion, DHLA, bisnorlipoic acid, β-hydroxybisnorlipoic acid and tetranorlipoic acid may contribute to the antioxidant effect of lipoic acid *in vivo*. As will be described, the antioxidant properties of DHLA have been studied extensively, the antioxidant properties of bisnor- and tetranorlipoic acid have been studied only occasionally and the other metabolites have not been studied.

METAL CHELATION

The chemistry

Metal chelation is a property of a compound that can result either in antioxidant or in pro-oxidant activity. Antioxidant activity is obtained when a complex is formed in which the metal is shielded and all coordination sites for O_2 are occupied. In addition, antioxidant activity is obtained when electron density is withdrawn from the metal to the chelator, so electrons cannot be transferred to O_2. Pro-oxidant activity is obtained when coordination sites for O_2 are present and the metal is reduced. The ligand transfers electrons to the metal, and the electrons are subsequently transferred to O_2. The most important transition metal in oxidative stress is iron. Studying iron complexation (Fe^{2+} or Fe^{3+}) by antioxidants in aqueous solutions at pH 7.4 is hampered by the formation of insoluble iron hydroxides. Therefore, iron complexation in an aqueous environment is studied mostly by displacing a known iron chelator.

In polar but nonaqueous solvents, it was shown that lipoic acid forms complexes with Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} (Sigel, 1982; Sigel and Prijs, 1978). In addition, lipoic acid does not chelate Fe^{3+} (Cornaro *et al.*, 1985). DHLA chelates Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+} (Sigel, 1982), Hg^{2+} (Brown and Edwards, 1970) and Fe^{3+} (Cornaro *et al.*, 1985; Kawabata *et al.*, 1995), resulting in complexes poorly soluble in water. Evidence has been produced that the DHLA complex with Fe^{3+} is more stable than that with Fe^{2+} (Bonomi *et al.*, 1985). The metabolites tetranor- and bisnorlipoic acid can form complexes with Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} (Sigel, 1982).

Metal chelation in vitro *and* in vivo

Lipoic acid may provide antioxidant activity by chelation of iron. This conclusion is based on results in an ·OH scavenging assay in which deoxyribose was used as a detector molecule (Scott *et al.*, 1994). Deoxyribose binds to iron, inducing site-specific degradation of deoxyribose. However, after addition of lipoic acid, it was concluded that lipoic acid displaces the deoxyribose from the deoxyribose-iron complex. It had been shown previously that lipoic acid does not chelate Fe^{3+} (Cornaro *et al.*, 1985). Therefore, it can be deduced that lipoic acid chelates Fe^{2+}, thus diminishing the amount of ·OH detectable by deoxyribose. In a lipid peroxidation test, indications for iron chelation also have been obtained. In this test, oxidative stress is induced by incubation of $Fe^{(2+ \text{ or } 3+)}$ and vitamin C. The vitamin C chelates the iron and reduces it to Fe^{2+}. Subsequently, Fe^{2+} can transfer one electron to oxygen or to other ROS and induce oxidative stress. At equimolar amounts of iron and vitamin C, lipoic acid is able to compete with vitamin C for chelation, and consequently protection against peroxidation of lipids was observed (Scott *et al.*, 1994). However, when vitamin C is in a 50-fold excess of iron, lipoic acid is unable to compete with vitamin C for chelation, and consequently no prevention of lipid peroxidation is observed (Fig. 7) (Bast and Haenen, 1988). In the same manner, complexation of Cu^{2+} by lipoic acid can explain protection in Cu^{2+}-induced lipid peroxidation (Ou *et al.*, 1995).

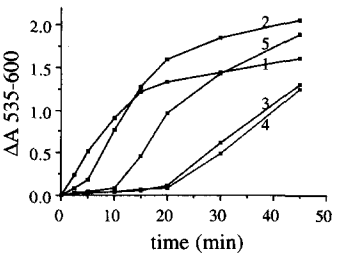


FIGURE 7. The time course of lipid peroxidation. By measuring thiobarbituric acid (TBA) reactive material, lipid peroxidation was assayed. After reaction with TBA, products are formed that absorb at 535 nm (at 600 nm is corrected for protein precipitation). Rat hepatic microsomes were incubated with 0.2 mM vitamin C and (1) no addition, (2) 0.5 mM DHLA, (3) 1 mM GSH, (4) 0.5 mM DHLA plus 1 mM GSH and (5) 0.5 mM DHLA plus 0.5 mM oxidized glutathione (GSSG). Addition of 0.5 mM GSSG or 0.5 mM lipoic acid alone had no effect, and the result was identical with that of time course (1). The effect of adding 0.5 mM lipoic acid plus 1 mM GSH did not differ from that obtained with 1 mM GSH alone (3). The last results are not indicated for the sake of clarity. All reactions were started with 10 μM Fe^{2+}.

The complexation of metals by DHLA also may result in antioxidant activity. This was suggested for lipid peroxidation induced by Cd^{2+} (Müller and Menzel, 1990). However, DHLA is also an effective reducing agent for some transition metals, resulting in pro-oxidant activity. For example, DHLA can easily reduce Fe^{3+} to Fe^{2+}. This reduction increases the amount of Fe^{2+} and consequently promotes (Fe^{2+}/vitamin C)-induced lipid peroxidation (Bast and Haenen, 1988; Scott *et al.*, 1994). This pro-oxidant activity is seen in Fig. 7 as a higher final level of TBARS compared with the control. Of course, one should be aware that pro-oxidant activity may be derived from a combination of Fe^{3+} reduction and vitamin C regeneration (described later). In general, DHLA has to compete with other chelating molecules for metals. *In vitro*, it was shown that excess DHLA could effectively compete with ferritine, thereby liberating iron from ferritine (Bonomi and Pagani, 1986), but it is not known whether this occurs *in vivo*, too. The ability of a compound to accelerate DNA degradation by Fe^{3+}/bleomycin is often used as a measure of its iron-reducing pro-oxidant activity. However, DHLA did not promote (Fe^{3+}/bleomycin)-induced DNA degradation (Scott *et al.*, 1994), indicating that it did not displace bleomycin from the complex.

In vivo indications for antioxidant activity through metal chelation were found in rats. Lipoic acid prevented (Cd^{2+})-induced lipid peroxidation in brain, heart and testes (Sumathi *et al.*, 1994).

ROS SCAVENGING CAPACITY

The chemistry

One method for modulating oxidative stress is to use ROS scavengers. Antioxidant activity is obtained when the reaction between the ROS and the scavenger is faster than the reaction between the ROS and target molecule (Fig. 8). In an organism, the many biomolecules present in tissue and in the intra- and extracellular fluids can be targets of ROS-mediated damage. As in all chemical reactions, the reaction rate of a scavenging reaction is determined by the concentration of the scavenger and by the rate constant (k_3 in Fig.

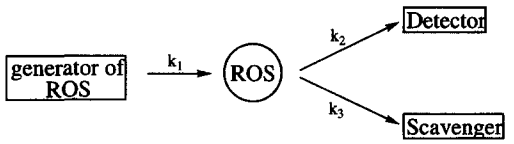


FIGURE 8. The principle of ROS scavenging. *In vivo*, endogenous biomolecules take the place of the detector molecule.

8). In test-tube experiments designed to test the ROS scavenging capacity of a compound, information about this rate constant is obtained. Two procedures have been applied to examine ROS scavenging capacity: a competition experiment and monitoring of the time course of concentration of a reactant. To illustrate the different tests, the principles of the hypochlorous acid (HOCl) scavenging tests are discussed.

In analogy to the principle of ROS scavenging, *in vivo* experiments can be designed as a competition assay, as shown in Fig. 8. In this assay, the scavenger competes with the detector molecule for the ROS. In the absence of the scavenger, the detector molecule produces a signal that is measured. Scavenging activity of a test compound is revealed by a suppression of the signal produced by the detector molecule. In many scavenging tests, the ROS under investigation is an unstable species and must be generated in the test tube. HOCl can be generated by the myeloperoxidase/H_2O_2/Cl system. However, HOCl is relatively stable and commercially available. Therefore, the test can be simplified by using HOCl instead of the HOCl generator (Winterbourn, 1985). The experiment starts with the addition of HOCl to a mixture of the scavenger and the detector molecule (Fig. 8). It should be noted that the concentrations of the scavenger and detector molecule are chosen in such a way that the amount of detector molecule is easily determined by the analytical equipment. The concentrations do not necessarily have to be in the physiological range. Because no rate constant is known for the reaction between HOCl and a suitable detector molecule, the rate constant for the scavenging reaction (k_3, Fig. 8) cannot be calculated. Therefore, the HOCl scavenging capacity of compounds is given as a rank order.

Recently, Folkes *et al.* (1995) tried to obtain rate constants by the second method (i.e., monitoring the time course of concentration of a reactant). They further simplified the HOCl scavenging test by omitting the detector molecule. Consequently, they dealt solely with the HOCl scavenging reaction. The rate of this reaction was determined by following one of the reactants: HOCl, the scavenger or the oxidized scavenger. It appeared, however, that efficient scavengers (e.g., GSH) react too fast with HOCl to determine their rate constants. Nevertheless, the principle of this method was successfully applied with other ROS (Tables 2 and 3).

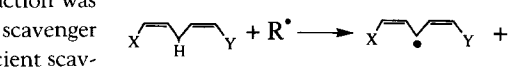
DHLA, lipoic acid, bisnorlipoic acid and tetranorlipoic acid have been tested in several HOCl scavenging assays. All compounds appeared to be good scavengers in the elastase assay (Biewenga *et al.*, 1994; Haenen and Bast, 1991; Scott *et al.*, 1994). In this assay, the detector molecule is α_1-antiprotease (α_1-AP). After reaction with HOCl, the remaining α_1-AP is determined as the remaining elastase inhibitory capacity of the mixture (Fig. 9). In the TNB assay, the thiol 5-thio-2-nitrobenzoic acid (TNB) is the detector molecule (Ching *et al.*, 1994). Only lipoic acid was tested in this assay, and it protected TNB more efficiently than did other scavengers such as S-methylglutathione (GSMe). DHLA was not tested because it interferes by reacting with oxidized TNB. In the protein carbonyl assay, oxidation of bovine serum albumin (BSA) by HOCl produces protein carbonyl groups that are detected by 2,4-dinitrophenylhydrazine (Yan *et al.*, 1996). DHLA appeared to be the most efficient scavenger of the compounds tested. Remarkably, lipoic acid was less efficient in the prevention of protein carbonyl formation than GSMe. The difference in rank order in HOCl scavenging activity found in the TNB and carbonyl assays may be explained by interfering reactions. Thiols and amines can interfere in the formation of protein carbonyl groups or by reaction of these compounds with the carbonyls (O'Neill *et al.*, 1994). In general, interference of test compounds on signal formation is one of the pitfalls of the competition assay.

Altogether, HOCl scavenging tests have been designed to obtain information about one reaction; that is, the reaction between HOCl and scavenger. Three comments can be made in this regard. First, it should be stressed that ROS scavenging is a matter of reaction rate. This is illustrated by β-lipoic acid. Although β-lipoic acid is able to react with HOCl, in the elastase assay, the reaction rate is not high enough to compete with α_1-AP (Biewenga *et al.*, 1994). Second, *in vivo*, the HOCl scavenging reaction never takes place as a single reaction but is always accompanied by subsequent reactions. The difference between the TNB and protein carbonyl assays may be explained by these reactions. This observation leads to the third remark. It nicely shows that antioxidant activity is a relative concept. TNB is protected by lipoic acid, whereas BSA is not. Thus, strictly speaking, the target molecule has to be considered when an antioxidant activity is ascribed to a compound.

Lipoic acid and its metabolites can also scavenge many other ROS. In Tables 2 and 3, an overview of the ROS scavenging capacities of lipoic acid and DHLA are given. For a complete discussion of the assays and their results, the reader is referred to other papers (Halliwell, 1995; Packer *et al.*, 1995; Scott *et al.*, 1994).

ROS scavenging in vitro

Oxidative stress is caused not by a single reaction but by a whole array of radical and nonradical reactions. Finally, oxidative stress can result in the destruction of proteins, DNA or lipids. Several tests have been developed to study the different oxidative processes. The most extensively studied oxidation is the lipid peroxidation process (Halliwell and Gutteridge, 1989). This process can be subdivided in four events (Fig. 10): the initiation reaction, the lipid hydroperoxide–independent and lipid hydroperoxide–dependent reactions and the termination reactions. The initiation reaction consists of an H· abstraction from a polyunsaturated fatty acid in the lipid membrane:



(15)

Subsequently, in a chain of reactions, more and more lipid-derived radicals are formed. Finally, this chain of reactions can stop when two radicals form a stable nonradical product.

In the lipid peroxidation process, two mechanisms of ROS scavenging lead to an antioxidant activity. First, a compound can scavenge the ROS that initiates the oxidative process. The chemical reactions behind this mechanism of ROS scavenging were described in the preceding paragraph. Second, a compound can act as a chain-breaking antioxidant. Vitamin E, for example, is an endogenous chain-breaking antioxidant. It reacts more rapidly with the intermediate radicals (the peroxy and alkoxy radicals in Fig. 10) than that these radicals can abstract H· from the surrounding lipids.

The effect of lipoic acid and DHLA in microsomal lipid peroxidation is shown in Fig. 7. The experiment was started by the generation of ROS in a Fe^{2+}–vitamin C mixture. In this test, antioxidant activity results in a lag time between the generation of the ROS and the beginning of the peroxidation. However, both lipoic acid and

TABLE 2. ROS scavenging of lipoic acid

Oxidant	Effect	Generator	Detector molecule	Detected reactant	Scavenging capacity	Reference
O_2^-	−	Xanthine oxidase	DMPO spin trap	—	—	(Suzuki *et al.*, 1991)
	−	Hypoxanthine oxidase	Cytochrome C	—	—	(Scott *et al.*, 1994)
H_2O_2	−	—	Peroxidase	—	—	(Scott *et al.*, 1994)
•OH	+	H_2O_2/Fe^{2+}	DMPO spin trap	—	DHLA = LA	(Suzuki *et al.*, 1991)
	+	H_2O_2/Fe^{2+}	Luminol	—	—	(Suzuki *et al.*, 1991)
	+	H_2O_2/Fe^{3+}/ascorbate	Deoxyribose	—	$k = 4.71 \times 10^{10}$/M/sec	(Scott *et al.*, 1994)
	+	Illumination NP-III	DMPO	—	$k = 1.92 \times 10^{10}$/M/sec	(Matsugo *et al.*, 1995)
	+	Illumination NP-III	BSA	—	—	(Matsugo *et al.*, 1995)
	+	Illumination NP-III	Salicylic acid	—	—	(Matsugo *et al.*, 1995)
	+	Illumination NP-III	Apo-B-protein	—	—	(Matsugo *et al.*, 1995)
HOCl	+	—	α_1-AP	—	DHLA, GSH, LA > GSMe > GSSG, cystine	(Haenen and Bast, 1991) (Biewenga *et al.*, 1994)
	+	—	TNB	—	LA > GSMe > GSSG	(Ching *et al.*, 1994)
	+	—	BSA	—	DHLA > GSH > GSMe > LA > cystine, GSSG	(Yan *et al.*, 1996)
CCl_3O_2•	+	Radiolysis CCl_4	—	CCl_3O_2•	$k = 1.8 \times 10^8$/M/sec	(Scott *et al.*, 1994)
$^1\Delta gO_2$	+	Rubene autoperoxidation	—	Rubrene	$k = 1 \times 10^8$/M/sec (benzene)	(Stevens *et al.*, 1974)
	+	Thermolysis $NDPO_2$	—	$^1\Delta gO_2$	$k = 1.38 \times 10^8$/M/sec	(Kaiser *et al.*, 1989)
$ONOO^-$	+	—	Tyrosine	—	DHLA, GSH > LA > GSSG	(Whiteman *et al.*, 1996)
	+	—	α_1-AP	—	DHLA, LA, Met > GSH > GSSG	(Whiteman *et al.*, 1996)
ABAP•	−	Thermolysis ABAP	B-phycoerythrin	—	—	(Kagan *et al.*, 1992)

Abbreviations: α_1-AP, α-1-antiprotease; ABAP, 2,2′-azobis(2-amidinopropane)dihydrochloride; BSA, bovine serum albumin; DHLA, dihydrolipoic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; GSMe, *S*-methylated glutathione; LA, lipoic acid; $NDPO_2$, endoperoxide of 3,3′-(1,4-naphthylide)dipropionate; NP-III, *N*,*N*′-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthalene-tetracarboxylic diimide); TNB, 5-thio-2-nitrobenzoic acid.

TABLE 3. ROS scavenging of DHLA

Oxidant	Effect	Generator	Detector molecule	Detected reactant	Scavenging capacity	Reference
O_2^-	+	Xanthine oxidase	DMPO spin trap	—	$k = 3.3 \times 10^5$/M/sec	(Suzuki *et al.*, 1991)
	+	Xanthine oxidase	Epinephrine	—	$k = 7.3 \times 10^5$/M/sec	(Suzuki *et al.*, 1993)
	−	Hypoxanthine oxidase	NBT	—		(Scott *et al.*, 1994)
	+	CytP450/adriamycin	DMPO spin trap	—	$k = 4.8 \times 10^5$/M/sec	(Dikalov *et al.*, 1996)
	+	CytP450/adriamycin	—	DHLA	$k = 4.8 \times 10^5$/M/sec	(Dikalov *et al.*, 1996)
H_2O_2	−	—	—	H_2O_2	—	(Haenen *et al.*, 1990)
	−	—	—	DHLA	—	(Scott *et al.*, 1994)
•OH	+	H_2O_2/Fe^{2+}	DMPO spin trap	—	DHLA = LA	(Suzuki *et al.*, 1991)
	−	H_2O_2/Fe^{3+}/vitamin C	Deoxyribose	—	—	(Scott *et al.*, 1994)
HOCl	+	—	α_1-AP	—	DHLA, GSH, LA > GSMe > GSSG, cystine	(Haenen and Bast, 1991) (Biewenga *et al.*, 1994)
	+	—	BSA	—	DHLA > GSH > GSMe > LA > cystine, GSSG	(Yan *et al.*, 1996)
CCl_3O_2•	+	Radiolysis CCl_4	—	CCl_3O_2•	$k = 2.7 \times 10^7$/M/sec	(Scott *et al.*, 1994)
$^1\Delta gO_2$	−	Thermolysis $NDPO_2$	—	$^1\Delta gO_2$	—	(Kaiser *et al.*, 1989)
	+	Photolysis duroquinone	—	$^1\Delta gO_2$	$k = 5.7 \times 10^5$/M/sec	(Bisby *et al.*, 1996)
NO•	+	Macrophages	—	NO• (as NO_2^-)	—	(Burkat *et al.*, 1993)
$ONOO^-$	+	—	Tyrosine	—	DHLA, GSH > LA > GSSG	(Whiteman *et al.*, 1996)
	+	—	α_1-AP	—	DHLA, LA, Met > GSH > GSSG	(Whiteman *et al.*, 1996)
ABAP•	+	Thermolysis ABAP	B-phycoerythrin	—	—	(Kagan *et al.*, 1992)

Abbreviations: α_1-AP, α-1-antiprotease; ABAP, 2,2′-azobis(2-amidinopropane)dihydrochloride; BSA, bovine serum albumin; DHLA, dihydrolipoic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; GSMe, *S*-methylated glutathione; LA, lipoic acid; NBT, nitro-blue tetrazolium; $NDPO_2$, endoperoxide of 3,3′-(1,4-naphthylide)dipropionate; TNB, 5-thio-2-nitrobenzoic acid.

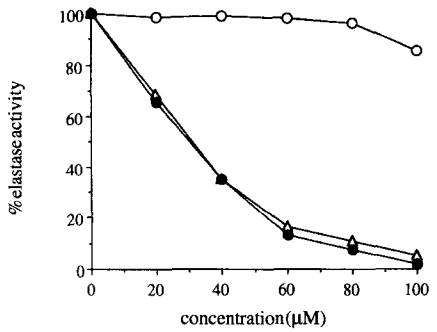


FIGURE 9. The HOCl scavenging capacity of DHLA (triangles), lipoic acid (solid circles) and β-lipoic acid (open circles). The test compounds were mixed with 20 μg of α_1-AP before 50 μM of HOCl was added. The remaining amount of detector molecule, α_1-AP, was expressed as its physiological functioning. The percentage of elastase inhibition was determined by using *N-t*-BOC-*L*-alanine *p*-nitrophenolester as a substrate. The rate of formation of the cleavage product was observed at 410 nm.

DHLA did not produce a lag time, indicating that they do not act as an antioxidant in this test. From this result, it is concluded that both compounds are not able to scavenge the initiating ROS produced by Fe^{2+}/vitamin C. In addition, the results indicate that lipoic acid and DHLA do not act as a chain-breaking antioxidant.

When the lipid peroxidation is initiated by thermolysis of 2,2'-azobis(2,4-dimethyl-valeronitrile (AMVN), a different result is obtained (Table 4). In this test, lipoic acid was a moderate antioxidant and DHLA a good antioxidant (Kagan *et al.*, 1992). This can be explained by prevention of the initiation of the lipid peroxidation process. DHLA probably scavenges the AMVN radical, which initiates the oxidative process because it was observed that it scavenges the structurally related ABAP radical (Table 3).

The different effects in the Fe^{2+}-vitamin C and AMVN-induced lipid peroxidation again illustrate the relativity of the term "antioxidant" Besides the importance of the kind of target molecule, the kind of oxidative process plays a role. In most *in vitro* ROS scavenger tests, the oxidative process is somehow related to an etiology. Central in those tests is the oxidizable substrate (e.g., the proteins, lipids, DNA, cell or organ).

For lipoic acid and DHLA, whether ROS scavenging tests can result in antioxidant activity has been examined (Table 4). Depending on the kind of oxidative stress and the kind of tissue that should be protected, it has been concluded that both lipoic acid and DHLA can act as antioxidant by scavenging ROS in *vitro*.

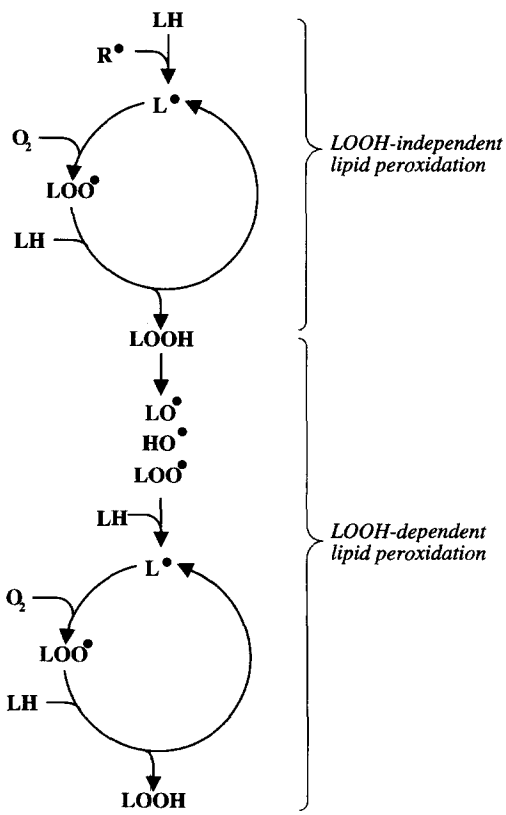


FIGURE 10. Sequence of reactions in lipid peroxidation. LH is a polyunsaturated fatty acid, and LOOH the corresponding lipid hydroperoxide.

ROS scavenging in vivo

Several tests have been performed to determine the antioxidant activity of lipoic acid and DHLA *in vivo* (Table 5). Most *in vivo* tests consist of an animal model for a certain disease of which it is assumed that oxidative stress accompanies its etiology. For example, streptozotocin is used in animal models for diabetes. It induces high blood glucose levels, which subsequently induce oxidative stress. So the nature of oxidative stress is more or less related to the disease process. However, oxidative stress can also be induced chemically. For example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Table 5) and alloxan (Fig. 11) have been used. In these cases, it should be noted that the nature of oxidative stress is unrelated to the disease. If, in chemically induced oxidative stress, antioxidant activity is observed, it only means that the compound effectively reaches the tissue to protect it from the chemically induced oxidative stress.

In vivo antioxidant activity can be determined by the measurement of: (1) the antioxidant levels, (2) the degree of damage (e.g., to lipids, proteins) and (3) a physiological function. The first point will be discussed in the paragraph about regeneration. As seen in Table 5, the amount of damage was measured by the determination of lipid peroxidation products, enzyme activity or cell viability. The third point, the prevention of the loss of a physiological function is, of course, the final goal of an antioxidant therapy. Only limited information about the molecular mechanism is obtained. For lipoic acid, protection of different pharmacological functions has been studied. For example, the glucose level indicates the functioning of β-cells, the dopamine level indicates the function of dopamine neurons and locomotor activity is related to the function of forebrain neuronal networks (see also Table 5).

In all tests, it is impossible to determine the mechanism of antioxidant activity—that is, by metal chelation, ROS scavenging, endogenous antioxidant regeneration or repair of oxidative damage. If specific products are generated in the reaction between ROS and antioxidant, the presence of these products *in vivo* provides evidence for ROS scavenging *in vivo*. For example, reaction products that are formed specifically in the ROS scavenging reaction have been analyzed *in vivo* for salicylic acid (Grootveld and Halliwell, 1986). For lipoic acid, β-lipoic acid is a ROS scavenging product (Biewenga *et al.*, 1994; Stary *et al.*, 1975). Therefore, measurement of β-lipoic acid may elucidate whether ROS scavenging took place in antioxidant activity. β-Lipoic acid formation has not yet been examined in *in vivo* antioxidant tests.

TABLE 4. ***In vitro*** **antioxidant activity of lipoic acid and DHLA**

Generation of oxidative stress by	Product to be protected	Measurement	Effect of lipoic acid	DHLA	Reference
$^1\Delta gO_2$ generation by thermolysis of $NDPO_2$	pBR322-DNA	Single-strand breaks	+	n.d.	(Devasagayam *et al.*, 1993)
Myoglobin/H_2O_2	Arachidonic acid	Lipid peroxidation	−	+	(Scott *et al.*, 1994)
Thermolysis of AMVN	Liposomes,	Lipid peroxidation	−	+	(Kagan *et al.*, 1992)
	tissue homogenates,		n.d.	+	
	microsomes		(±)*	+	
Fe^{2+}/vitamin C	Microsomes	Lipid peroxidation,	−	−	(Bast and Haenen, 1988)
		Ca^{2+}-ATPase activity	n.d.	+	(Pruijn *et al.*, 1991)
ADP/Fe^{3+}/NADPH	Microsomes	Lipid peroxidation	−	+	(Scholich *et al.*, 1989)
Fe^{3+}/vitamin C	Liposomes	Lipid peroxidation	+	−	(Scott *et al.*, 1994)
Addition of O_3	Blood plasma	Protein oxidation,	n.d.	−	(van der Vliet *et al.*, 1995)
		lipid peroxidation,	n.d.	−	
		vitamin C level	n.d.	+	
Cigarette smoke	Plasma proteins	Creatine kinase activity	n.d.	!	(Scott *et al.*, 1994)
Cu^{2+}/H_2O_2/epinephrine	LipDH	LipDH activity	−	+	(Gutierrez Correa and Stoppani, 1996)
H_2O_2 addition	Rat heart mitochondria	Lipid peroxidation	n.d.	+	(Scheer and Zimmer, 1993)
Cd^{2+}	Hepatocytes	Cell viability,	+	n.d.	(Müller, 1989)
		sorbitol dehydrogenase activity,	+	n.d.	
		lipid peroxidation	+	n.d.	
Activated macrophages	Islet cells	Cell lysis	n.d.	+	(Burkat *et al.*, 1993)
Hypoxanthine oxidase	Islet cells	Metabolic activity	n.d.	+	(Burkat *et al.*, 1993)
Ischemia	Perfused Langendorff hearts	Heart function	n.d.	−	(Haramaki *et al.*, 1993)
Ischemia	Perfused Langendorff hearts**	Heart function,	+	n.d.	(Serbinova *et al.*, 1992)
		cell viability,	+	n.d.	
		protein oxidation	+	n.d.	
Hypoxia/reoxygenation	Isolated rat heart	Aortic flow	+	n.d.	(Zimmer *et al.*, 1995)
TNF-α and PKC stimulation of T cells	Transcription factor NF-κB present in living T cells	Dissociation of NF-κB,	+	+	(Suzuki and Packer, 1994)
		NF-κB-mediated gene expression,	+	+	
		ATPase activity	+ (R-isomer)	n.d.	
Irradiation	Murine tumor cells	Cell viability,	+	n.d.	(Busse *et al.*, 1992)
		antioxidant (GSH) status	+	n.d.	

Abbreviations: +, antioxidant activity; −, no antioxidant activity; !, pro-oxidant activity; AMVN, 2,2′-azobis(2,4-dimethyl-valeronitrile); LipDH, lipoamide dehydrogenase; n.d., not determined; $NDPO_2$, endoperoxide of 3,3′-(1,4-naphthylide)dipropionate; PKC, protein kinase C; TNF, tumor necrosis factor α; (±)*, ca 15% inhibition of the lipid peroxidation, not concentration dependent; **, hearts from animals fed with lipoic acid.

REGENERATION OF ENDOGENOUS ANTIOXIDANTS

The chemistry

Scavenging of ROS, especially radicals, is efficient only when it forms a relatively stable oxidation product that can be safely regenerated or degraded. Glutathione and vitamin C are endogenous molecules that take part in the regeneration of oxidized antioxidants. Through a cooperative set of reactions, the different antioxidants interact with each other. For example, after scavenging, the vitamin C radical is formed, leading to the following interplay of antioxidants (the name of the enzyme that might catalyze the reaction is given parenthetically):

$$2\ \text{vit C}^{\bullet} \rightarrow \text{vit C} + \text{DHAA}\ (\text{nonenzymatically}) \quad (16)$$

$$\text{DHAA} + 2\ \text{GSH} \rightarrow \text{GSSG} + \text{vit C}\ (\text{GSH-dehydroascorbate reductase}) \quad (17)$$

$$\text{NADH} + 2\ \text{vit C}^{\bullet} \rightarrow \text{NAD}^{+} + 2\ \text{vit C}\ (\text{NADH-semidehydroascorbate reductase}) \quad (18)$$

The vitamin E radical also can be regenerated. The following stoichiometric reactions have been described:

$$2\ \text{vit E}^{\bullet} + 2\ \text{GSH} \rightarrow 2\ \text{vit E} + \text{GSSG}\ (\text{free radical reductase}) \quad (19)$$

$$2\ \text{vit E}^{\bullet} + \text{vitC} \rightarrow 2\ \text{vit E} + \text{DHAA}\ (\text{nonenzymatically}) \quad (20)$$

Additionally, the GSSG formed in these reactions can be reduced back to GSH:

TABLE 5. *In vivo* antioxidant activity of lipoic acid and DHLA

Species	Generation of oxidative stress by	Measurement	Effect of Lipoic acid	Effect of DHLA	Relation to illness	Reference
Human	HIV infection	Endogenous antioxidant levels,	+	n.d.	AIDS	(Fuchs *et al.*, 1993)
			+	n.d.		
		T helper lymphocytes,	+	n.d.		
		lipid peroxidation products				
Rat	Glyoxylate	Antioxidant levels,	+	n.d.	Renal lithiasis	(Sumathi *et al.*, 1993)
		lipid peroxidation	+	n.d.		
Rat	Glyoxylate	Na/K-ATPase activity of erythrocytes,	+	n.d.	Renal lithiasis	(Jayanthi and Varalakshmi, 1992)
			+	n.d.		
		Ca-ATPase activity of erythrocytes,	−	n.d.		
		phospholipid composition				
Rat	Ethanol intoxication	Ethanol plasma level,	+	n.d.	Ethanol intoxication	(Xie *et al.*, 1994)
		sleep time	+	n.d.		
Gerbil	Forebrain ischemia/ reperfusion	Locomotor acitivity,	+	n.d.	Brain ischemia	(Cao and Phillis, 1995)
		hippocampal cell damage	+	n.d.		
Rat	Cerebral ischemia/ reperfusion	Mortality,	+	n.d.	Brain ischemia	(Panigrahi *et al.*, 1996)
		GSH level brain	+	n.d.		
Rat	Excitotoxicity by NMDA,	Histology striatum	+	+	Stroke, hypoglycemia,	(Greenamyre *et al.*, 1994)
	excitotoxicity by malonic acid		+	+	Huntington and Parkinson	
Mouse	Intradermal glucose oxidase, topical anthralin	Diameter inflammated area	+ (*R*, oral)	+ (intradermal)	Skin inflammation	(Fuchs and Milbradt, 1994)
			n.d.	+ (intradermal)		
Rat	MPTP	Vitamin E in striatal tissue,	−	n.d.	Parkinson	(Gotz *et al.*, 1994)
			+	n.d.		
		coenzyme Q, dopamine level in striatum	−	n.d.		
Rat	Streptozotocin	Nerve conduction,	+	n.d.	Diabetic polyneuropathy	(Nagamatsu *et al.*, 1995)
		endogenous antioxidants,	+	n.d.		
		nerve blood flow	+	n.d.		
Mouse	Irradiation	Amount of leukocytes, lymphocytes and platelets	+	n.d.	Irradiation damage	(Busse *et al.*, 1992)
Rat	Cd injection (s.c.)	Lipid peroxidation,	+	n.d.	Cd intoxication	(Sumathi *et al.*, 1996)
		antioxidant levels	+	n.d.		
Rat	Buthionine sulfoximine	Development of cataract,	+	n.d.	Cataract	(Maitra *et al.*, 1995)
		endogenous antioxidant levels	+	n.d.		

Abbreviations: +, antioxidant activity; −, no antioxidant activity; HIV, human immunodeficiency virus; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; n.d., not determined; NMDA, *N*-methyl-D-aspartate.

$$NADPH + GSSG \rightarrow NADP^+ + 2\ GSH \quad \text{(glutathione reductase)} \qquad (21)$$

Except for the reaction catalyzed by GSH-reductase, it is still a matter of debate which of the preceding reactions are catalyzed by enzymes. For example, the physiological relevance of the enzyme dehydroascorbate reductase is doubtful, because the vitamin C regeneration is only twofold to fivefold accelerated by the proposed protein (Winkler *et al.*, 1995). It may be proposed that one-electron reductions require "safe" protein surrounding of an enzyme. Indeed, there is less discussion about the existence of the enzymes that catalyze a one-electron reduction (the free radical reductase and NADH-semidehydroascorbate reductase) than about the dehydro-ascorbate reductase.

Lipoic acid, after reduction to DHLA, is able to contribute to the nonenzymatic regeneration of GSH and vitamin C.

$$DHLA + GSSG \rightarrow \text{lipoic acid} + 2\ GSH \qquad (22)$$

$$DHLA + DHAA \rightarrow \text{lipoic acid} + \text{vit C} \qquad (23)$$

The occurrence of these reactions has been confirmed by measurements of GSH formation (Bast and Haenen, 1988) and vitamin C formation (Haenen and Bast, 1989) in the presence of DHLA. For vita-

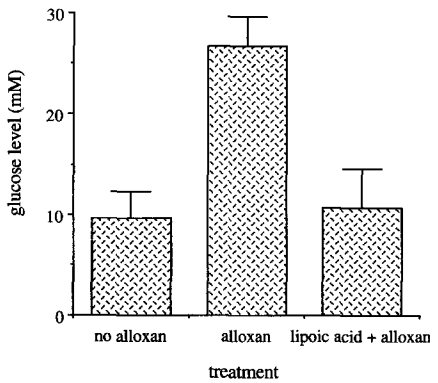


FIGURE 11. Plasma glucose levels 72 hr after treatment with alloxan (100 mg/kg, IV). Lipoic acid (100 mg/kg, IP) was given 30 min prior to the alloxan injection. The male SE outbred mice (ca. 30 g) fasted 4 hr before measurement of the glucose level in plasma derived from the tail veins. Data are shown as the mean of at least four experiments±SD.

min C regeneration, the rate constant has been estimated (Table 6). DHLA shows greater reactivity toward dehydroascorbic acid (DHAA) than does GSH, indicating that, at certain concentrations, DHLA is preferentially used for vitamin C regeneration over GSH.

Interestingly, DHLA regenerates not only endogenous antioxidants. It was shown that DHLA could regenerate ebselen selenol (Haenen *et al.*, 1990) and that the oxidation product of lipoic acid, β-lipoic acid, is reduced by DHLA (Fig. 12):

DHLA + β-lipoic acid → 2 lipoic acid (24)

In vitro *regeneration*

Two approaches are possible for studying the interplay of antioxidants *in vitro*. After addition of the proposed regenerating antioxidant, either the amount of the antioxidants or a synergistic protection against oxidative damage can be quantified.

The first approach was applied by Xu and Wells (1996); they studied the regeneration of vitamin C. They determined the amount of vitamin C formed by incubating rat liver mitochondria or mitoplasts with lipoic acid. In the presence of NADH, the addition of lipoic acid resulted in increased vitamin C levels. The necessity for NADH indicates that DHLA is the regenerating species:

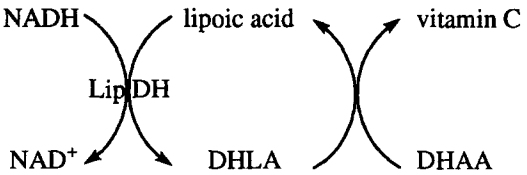
TABLE 6. Nonenzymatic regeneration of vitamin C

Reductor	Method*	*k* (M/min)	SD
GSH	1	32.8	1.0
DHLA	2	1603	300
	1	875	95

*(1) The rate constant was determined by following the rate of vitamin C formation at 265 nm. A concentration of (0.2–0.1 mM) dehydroascorbate was incubated with (0.35–0.50 mM) DHLA.

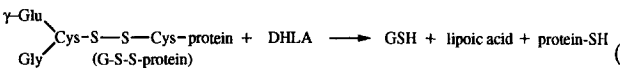
(2) The rate constant was determined from the steady-state concentration of vitamin C observed at 265 nm. A concentration of 0.25 μg/ml was incubated with ascorbate oxidase with 0.05 mM vitamin C and 1.0–1.5 mM DHLA.

Both reactions were performed at 37°C, 50 mM KH_2PO_4/KOH, pH 7.4.



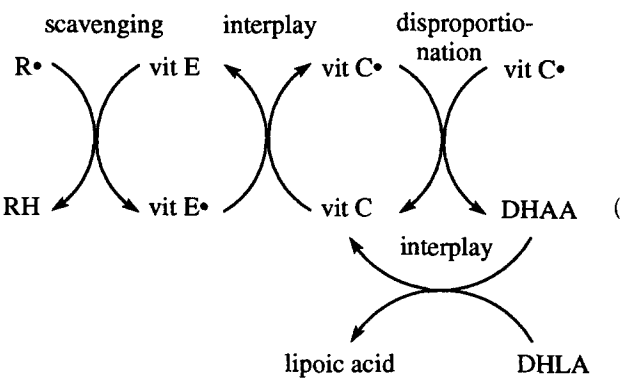
(25)

The regeneration of GSH also was studied by measuring GSH levels. Busse *et al.* (1992) observed an increase in the amount of GSH in murine neuroblastoma and melanoma cells after incubation with lipoic acid. Han *et al.* (1995) found similar results in human T-lymphocyte Jurkat cells. Remarkably, the elevation of GSH levels was larger than the amount of GSSG normally present in these cells. This may also indicate that liberation of GSH takes place from mixed disulfides [reaction (26)], although it may not be ruled out that lipoic acid stimulates the biosynthesis of glutathione by an unknown mechanism.



(26)

The second approach, studying the synergism in the prevention of oxidative damage, is best applied when the regenerating antioxidant alone does not contribute to overall antioxidant activity (Fig. 13). Such circumstances were found by Bast and Haenen (1988) and Kagan *et al.* (1992). Kagan *et al.* found that DHLA did not protect dioleoylphosphatidylcholine liposomes from ultraviolet-induced peroxidation. And, in another *in vitro* test, DHLA showed no protection from lipoxygenase-induced peroxidation in microsomes. After induction of the lipid peroxidation process, the electron spin resonance (ESR) signal of the chromanoxyl radical present in the lipid bilayer was an indication that the peroxidation was proceeding. After addition of vitamin C to the lipid suspension, the chromanoxyl radical disappeared and the ascorbyl radical appeared. Gradually, the ascorbyl radical decreased in time, and progressively, the chromanoxyl radical appeared. The interaction of DHLA with dehydroascorbate was seen as a longer lifetime of the ESR signal of the ascorbyl radical before the lipid peroxidation continued.



(27)

Bast and Haenen (1988) studied the interplay between DHLA and GSH. They observed synergism in the protection against Fe^{2+}-vitamin C–induced lipid peroxidation. As seen in Fig. 7, antioxidant activity is found for DHLA only when it is coincubated with GSSG.

DHLA does not regenerate the chromanoxyl radical present in vitamin E directly. This can be concluded from the study reported by Kagan *et al.* (1992). DHLA alone did not decrease the signal of the chromanoxyl radical in the liposomes. However, vitamin E can be regenerated by DHLA in a cascade of regenerating reactions.

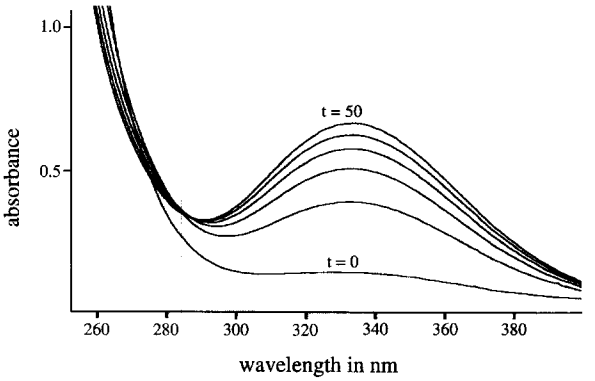


FIGURE 12. A concentration of 5 mM DHLA (spectrum $t=0$) was incubated with 5.16 mM β-lipoic acid at 37°C and pH 7.4. Every 10 min, a spectrum was recorded. The increase at 333 nm shows the formation of lipoic acid. The second-order rate constant was determined: $k=0.51\times10^{-2}$ M/min.

Scholich *et al.* (1989) reported a nonenzymatic regeneration of vitamin E by DHLA, independently of GSH. However, Bast and Haenen (1988) found that protection against lipid peroxidation by DHLA depended on GSH (Fig. 7). They proposed that the regeneration of GSH [reaction (22)] is followed by the enzymatic regeneration of vitamin E [reaction (19)].

In vivo *regeneration*

The two approaches used for *in vitro* tests have been applied to the study of the regeneration of antioxidants; that is, the measurement of antioxidant levels or the determination of synergistic protection. Vitamin C regeneration has been studied in healthy newborn rats. It was shown that the administration of lipoic acid did not statistically significantly affect the vitamin C levels in the eye lens (Maitra *et al.*, 1995). Vitamin C regeneration has also been studied in organisms subjected to oxidative stress. In plasma of HIV-positive patients, lipoic acid increased vitamin C levels (Fuchs *et al.*, 1993). In the eye lenses of newborn rats (Maitra *et al.*, 1995), lipoic acid prevented a decrease in vitamin C levels induced by BSO treatment. Clearly, lipoic acid can affect vitamin C levels. However, it is not known whether vitamin C regeneration occurs or whether lipoic acid acts as a scavenger, thus sparing vitamin C.

Regarding vitamin E, in healthy animals, no effect of lipoic acid administration was found on the vitamin E level (Maitra *et al.*, 1995; Nagamatsu *et al.*, 1995). BSO treatment of newborn rats resulted in a decreased vitamin E level, which is prevented by lipoic acid (Maitra *et al.*, 1995). Nagamatsu *et al.* (1995) studied the interplay between vitamin E, GSH and lipoic acid. Depletion of vitamin E, together with oxidative stress derived from streptozotocin-induced diabetes, resulted in decreased levels of GSH. However, this was not accompanied by an increase in GSSG. Moreover, lipoic acid did not prevent this GSH depletion, confirming that oxidized glutathione [or its mixed disulfide, reaction (26)] is necessary for the regeneration of GSH.

For GSH regeneration, different effects of lipoic acid have been reported in healthy subjects. In mice, total GSH levels were increased in liver, kidney and lung tissue cells after lipoic acid administration (Busse *et al.*, 1992). In contrast, lipoic acid did not affect the GSH or GSSG levels in the sciatic nerve of healthy rats (Nagamatsu *et al.*, 1995). In animals subjected to oxidative stress, lipoic acid prevented depletion of GSH in all reported experiments (Maitra *et al.*, 1995; Nagamatsu *et al.*, 1995; Sumathi *et al.*, 1993).

The second approach, synergistic prevention *in vivo*, was studied as early as 1959 by Rosenberg and Culik (1959). They concentrated on end-point parameters of vitamin C– and vitamin E–deficiency scurvy and reproduction failure, respectively. In vitamin C–deficient guinea pigs, suboptimal amounts of vitamin C in combination with lipoic acid prevented scurvy symptoms better than did either compound alone. For vitamin E-deficient animals, a similar synergistic protection was found. The combination of 15 mg of lipoic acid plus 25 mg of vitamin E was more effective than either one of the compounds alone against the symptoms of vitamin E deficiency.

Altogether, lipoic acid seems to regenerate vitamin C, vitamin E and GSH *in vivo*. For *in vivo* tests, one should keep in mind that it is difficult to determine whether a lessened decrease in antioxidant level is due to additional scavenging, to stimulated biosynthesis (as might occur with GSH) or to regeneration of another antioxidant by the drug. At least it is safe to state that none of the *in vivo* tests excluded regeneration of the endogenous antioxidants. Especially, the synergism found in the experiments of 1959 are a good indication that lipoic acid is able to regenerate vitamin C and vitamin E *in vivo*.

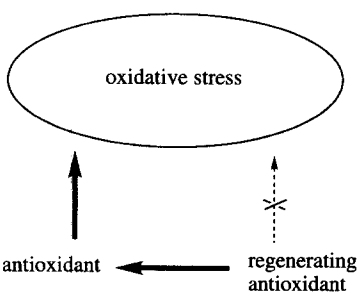
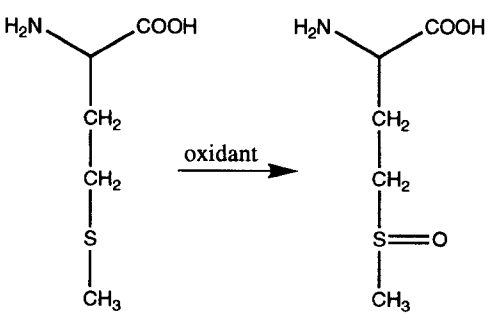


FIGURE 13. Regeneration of an antioxidant can be studied as synergism in the prevention of oxidative damage. Minor contribution of the regenerating antioxidant to the overall antioxidant activity is a necessary condition for this principle.

REPAIR OF OXIDATIVE DAMAGE: IMPROVEMENT OF PMSR ACTIVITY

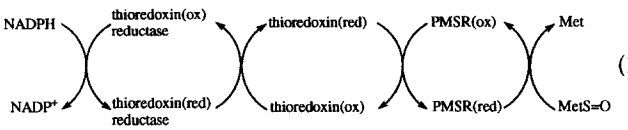
Oxidative stress may result in damage to DNA, lipids and proteins. One method of overcoming oxidative damage is degradation and renewal. A second method is repair, which may be particularly important for proteins with a low turnover rate. In the proteins, amino acid residues such as tryptophan, histidine, tyrosine, cysteine and methionine are susceptible to oxidation. Whereas some oxidants (e.g., ozone, superoxide anions/hydroxyl radicals) destroy the residues at random, other oxidants (e.g., H_2O_2, HOCl, chloramines and $ONOO^-$) preferentially oxidize exposed methionine residues (Maier *et al.*, 1989).



methionine (Met) methionine sulfoxide (MetS=O) (28)

Methionine oxidation of some residues does not affect physical or immunochemical properties or biological activity of the peptide at all, whereas oxidation of other, specific methionine residues immediately leads to inactivation of the protein. This is observed for several enzymes, hormones, chemotactic factors and plasma proteinase inhibitors. For these peptides, inactivation by methionine oxidation has been regarded as part of a regulatory, physiologic process (Swaim and Pizzo, 1988). A particular example of oxidative regulation is the regulation of the activity of proteinase inhibitors such as α_1-AP. Neutrophils, macrophages and other leukocytes secrete large quantities of oxidants at sites of inflammation and may readily bring about methionine oxidation, resulting in loss of protease inhibitory activity. Inactivation of proteinase inhibitors may alter the proteinase-antiproteinase balance in favor of the protein-degrading enzyme. Protein degradation facilitates phagocytosis of invading organisms but should be restricted to exogenous material. A poorly controlled proteinase-antiproteinase balance results in degradation of endogenous tissue, and this forms the basis for pathological processes such as as lung emphysema.

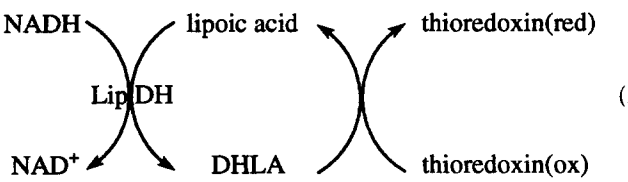
Two enzymes have been reported to be able to reduce oxidized other (PMSR) reduces peptide bound methionine sulfoxide. The enzyme PMSR requires reducing equivalents for its reaction. It is proposed that the thioredoxin system supplies the enzyme with electrons (Brot *et al.*, 1981):



(29)

Excessive accumulation of methionine sulfoxide residues can be caused by reduced PMSR activity. This decreased activity can originate from a decreased amount of PMSR or by a decreased amount of reducing equivalents (Brot and Weissbach, 1988).

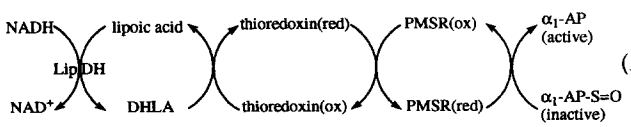
Lipoic acid can improve the repair of oxidized methionine residues by supplying PMSR with reducing equivalents. This can be done by increasing the amount of reduced thioredoxin. Holmgren (1979) and Spector *et al.* (1988) showed that dihydrolipoamide, an analogue of dihydrolipoic acid, can reduce thioredoxin in the following system:



(30)

This system can be coupled to PMSR and to the PMSR-dependent regeneration of α_1-AP. As shown in Fig. 14, DHLA can regenerate α_1AP by itself, but the reaction is catalyzed in the presence of partly purified PMSR from rat lungs and is further accelerated by thioredoxin.

When DHLA is derived from LipDH-dependent reduction, the ultimate effect of lipoic acid is making NADH available as a source for reductive reactions [reaction (31)] instead of NADPH [reaction (29)].



(31)

In vivo, the activity of PMSR is difficult to detect (Glaser *et al.*, 1987). Only recently, reversal of damage due to methionine oxidation was shown in living cells (Moskovitz *et al.*, 1995). Although the contribution of lipoic acid to protein repair still has to be demonstrated *in vivo*, the *in vitro* experiments provide evidence that lipoic acid participates in the repair of methionine residues by reduction of PMSR.

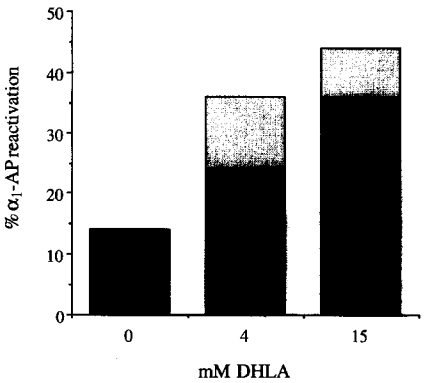


FIGURE 14. The repair of oxidatively damaged α_1-AP by DHLA (black) is accelerated by PMSR (dark gray) and is further accelerated by PMSR plus thioredoxin (light gray). PMSR was partly purified from Wistar rat lungs, by the method of Carp *et al.*, (1983) followed by anion exchange chromatography on a 26/10 Sepharose column. An amount of 200 μg α_1-AP was oxidized by 1.25 mM HOCl in a volume of 40 μl. The compounds were incubated with 37 units PMSR for 1 hr at 37°C in a volume of 280 μl. (One unit is defined as the percentage of reactivated α_1-AP per mg protein after 1 hr in the presence of 15 mM dithiothreotol (DTT) at 37°C.) The percentage of α_1-AP repair was determined in 850 μl, using 25 μg elastase and 60 μM N-succinyl-(Ala)$_3$-*p*-nitroanilide and assuming a perfect inverse relation between the amount of α_1-AP and elastase activity. The rate of formation of *p*-nitroaniline was observed at 410 nm.

PRO-OXIDANT ACTIVITY

Pro-oxidant activity is defined as the activity of an antioxidant in a situation where it produces more oxidative stress than if when it were absent. The ability of a compound to donate an electron to an oxidant can make that compound an antioxidant. Under different conditions, however, exactly the same ability can turn that compound into a pro-oxidant. This can be demonstrated by thiols. Usually thiols are considered to be antioxidants. However, thiols can also promote $O_2^{\bullet -}$ formation by direct reaction with O_2. Although $O_2^{\bullet -}$ generation by DHLA has not been reported, it is known that DHLA oxidizes by air, indicating that oxygen-reduction occurs. Generally, transition metals catalyze $O_2^{\bullet -}$ formation and other ROS-producing reactions. Indeed, Fe^{3+} also increases the rate of oxygen consumption by DHLA (Kawabata *et al.*, 1995), indicating that DHLA reduces Fe^{3+}, which subsequently reduces O_2. In addition, this one-electron reduction of a transition metal may also occur indirectly by the regeneration of vitamin C [reaction (23)]. Increased Fe^{3+} reduction can result in pro-oxidant activity. This was observed in two lipid peroxidation tests (Bast and Haenen, 1988; Scott *et al.*, 1994). Remarkably, lipoic acid inhibits the pro-oxidant activity of DHLA (Scott *et al.*, 1994).

In another route to pro-oxidant activity, the antioxidant reacts with a ROS scavenger, and a product is formed that is more harmful than the ROS that is scavenged. This has been observed with smoke-derived ROS and $^{\bullet}OH$ radicals. In smoke, ROS are present, inactivating creatine kinase. DHLA scavenges these ROS. However, the scavenging product more efficiently inactivates creatine kinase than does the original ROS (Scott *et al.*, 1994). Irradiation of an α_1-AP-containing solu-

tion results in $^{\bullet}OH$ formation and subsequent inactivation of α_1-AP. Relatively low concentrations of lipoic acid or DHLA accelerate this inactivation. This has been explained by the formation of thiyl and oxysulfur radicals after scavenging. Interestingly, at high concentrations, both lipoic acid and DHLA act as antioxidants (Scott *et al.*, 1994).

Altogether, DHLA can have pro-oxidant activity by reducing transition metals. In addition, both lipoic acid and DHLA can act as pro-oxidants by the formation of reactive scavenging products.

CONCLUSIONS

In this review, we considered drug-related antioxidant pharmacology. In pathology and physiology, oxidative processes take place. An imbalance between oxidative and antioxidative processes results in oxidative stress. Drugs can intervene in oxidative processes as antioxidants and delay or prevent their damaging effects. The term "antioxidant" depicts a relative concept. Antioxidant activity depends on which biological target molecules have to be protected from which kind of oxidative reactions. Antioxidant pharmacology related to a certain pathology can provide information into the nature of oxidative stress and the biological target molecules in the etiology. In drug-related antioxidant pharmacology, research concentrates on the the antioxidant activity of a certain compound. The antioxidant activity of a compound is determined by its antioxidant properties and by its concentration. Similar to other drugs, the metabolites may contribute to the overall antioxidant activity and pharmacological effect *in vivo*.

On the basis of lipoic acid, research concentrating on the antioxidant activity of a drug was discussed. Metabolites that contribute to the antioxidant activity of lipoic acid in *vivo* are DHLA and the β-oxidation products bisnor-, and tetranorlipoic acid. For DHLA and lipoic acid four antioxidant properties ware thoroughly examined: metal chelation, ROS scavenging, regeneration of endogenous antioxidants and repair of oxidatively damaged biomolecules. For the determination of these properties, test-tube experiments were performed, characterizing the chemical reactivity. The corresponding antioxidant activity was determined *in vitro* and *in vivo*. For the sake of clarity, a strict division was made between the different antioxidant properties and between the chemical *in vitro* and *in vivo* tests. In pathology, of course, all separate antioxidant mechanisms may proceed at the same time. The following properties were described for lipoic acid and its metabolites. Lipoic acid can have antioxidant activity by chelating Fe^{2+} and Cu^{2+}, and DHLA by chelating Cd^{2+}. DHLA can also chelate iron and reduce Fe^{3+} to Fe^{2+}, which results in pro-oxidant activity. Both lipoic acid and DHLA are scavengers of several ROS. Mostly, this results in antioxidant activity, except in three reported experiments in which the ROS-scavenging product appeared more damaging than the original ROS. (Remarkably, some pro-oxidant activity of DHLA can be inhibited by lipoic acid.) DHLA can participate in the regeneration of endogenous antioxidants such as vitamin C, GSH and vitamin E. In addition, DHLA may repair oxidative damage to proteins by improving the activity of PMSR.

Altogether, DHLA has more antioxidant properties than does lipoic acid. Therefore, it is likely that DHLA plays a major role in the observed antioxidant activity of lipoic acid *in vivo*. DHLA is formed by reduction of lipoic acid. Through LipDH-catalyzed reduction, the cell can draw on its NADH pool for antioxidant activity additionally to its NADPH pool, which is usually consumed during oxidative stress.

Antioxidant drugs can prevent the onset of pathologies, can delay pathological processes or may even take part in repair. Many experimental results show that both lipoic acid and DHLA can improve the antioxidant capacity of tissue against different forms of oxidative stress. At the moment, research on antioxidant drugs and research related to oxidative disease processes have not converged into a therapeutic intervention in which the mode of action is fully comprehended. However, research on the antioxidant pharmacology of the drug lipoic acid has given a stimulating impulse to antioxidant therapy.

We acknowledge D. H. Veening-Griffioen and Dr. H.-P. Voss, for testing lipoic acid in the alloxan-induced diabetes model. We thank A. J. Nicastia, who studied the regeneration of vitamin C by DHLA (Table 6). The recommendations of Dr. E. M. Mooijman regarding linguistic matters and the general comments of Dr. J. B. G. Paquay are highly appreciated.

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