ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 8, pp. 809-827. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 8, pp. 995-1017.

REVIEW

Free Radical Oxidation of Proteins and Its Relationship with Functional State of Organisms

V. I. Lushchak

Department of Biochemistry, Stefanyk Precarpathian National University, 57 Shevchenko Str., 76025 Ivano-Frankivsk, Ukraine; fax: (38-034) 223-1574; E-mail: lushchak@pu.if.ua

Received January 15, 2007
Revision received May 4, 2007

Abstract—Most reactive oxygen species (ROS) in living organisms are produced as byproducts of many processes. Being highly active, ROS interact with virtually all cellular components particularly modifying their properties. In this review, detailed analysis of chemical modifications of proteins on their interaction with ROS is given with particular interest in cleavage of polypeptide chains and oxidation of side chains of amino acid residues. Special attention has been paid to identification of products of free radical modification of proteins with a focus on the formation of additional carbonyl groups, which are the most frequently used markers of these processes. Functional consequences of protein modification by ROS depend on the nature of ROS and protein as well as particular conditions of their interaction. The relationship between protein oxidation and functional state of organisms, particularly aging, hyperoxia and hypoxia, and heat shock, as well as with different pathologies has been analyzed. The final part of the article is devoted to possible ways of protecting proteins against oxidation *in vivo*.

DOI: 10.1134/S0006297907080020

Key words: free radicals, protein oxidation, oxidative stress, aging, diabetes mellitus, atherosclerosis, neurodegenerative diseases

Although free radical processes in living systems have been studied for many years, interest in this problem has not decreased, but on the contrary the problem increasingly attracts the attention of researchers. This reflects the importance of the problems from the fundamental point of view and also because of involvement of these processes in many pathological states. And although in a very few cases the reactive species of oxygen (ROS), nitrogen (RNS), and/or sulfur (RSS) are known to be responsible for the development of pathologies, their increased concentrations are associated with many diseases such as ischemia/reperfusion, atherosclerosis, neurodegenerative diseases, diabetes mellitus, etc. Most studies have been focused on ROS, but from the end of the 1970s and in the beginning of the 1980s, when nitric oxide ($^{\bullet}NO$) was identified as a regulator of many cellular functions, the number of studies in the area have increased in geometric progression. The investigation of reactive species of sulfur and carbon started recently and very few aspects are disclosed, but even now there are no doubts of their importance in cellular functions.

Reactive oxygen species include superoxide anion ($O_2^{\bullet-}$), peroxides of hydrogen (H_2O_2) and other compounds (ROOH), hydroxyl radical ($^{\bullet}OH$), etc. The group of RNS consists of nitric oxide ($^{\bullet}NO$) and its derivatives, for example, peroxynitrite ($OONO^-$). Thiyl radical ($RS^{\bullet}$) and its derivatives are examples of reactive sulfur species. All the compounds listed above are very active from a chemical point view. There is much information on production of reactive species in cells, and they are described in detail by many authors [1-4]. Soviet researchers played a critical role in elucidation of mechanisms of free radical modification of lipids. The effects caused by free radical oxidation of DNA by reactive oxygen and nitrogen species were also studied in detail. However, one more group of biopolymers, proteins,

Abbreviations: CP) protein carbonyl groups; GAPD) glyceraldehyde-3-phosphate dehydrogenase; GS) glutamate synthase; HNE) 4-hydroxy-2-nonenal; MDA) malondialdehyde; MeSOX) methionine sulfoxide; OXDH) dehydrogenases of 2-oxo acids; RNS) reactive nitrogen species; ROS) reactive oxygen species; RSS) reactive sulfur species; TBARS) thiobarbituric acid-reactive substances.

which are also attacked by reactive species, started to receive enough attention only in the last 15-20 years. Since in the Russian language literature the problem is not highlighted enough, we would like to call attention of readers to this area. One may suggest that free radical modification of proteins is not very critical for cell survival as is oxidation of lipids, which may cause cell lysis, or DNA, which may result in mutations, but this is not the case. It is important to note that modification of proteins induced by free radicals can result in the loss of their functions, but also that living organisms use them for intra- and intercellular communication and to obtain information from the environment.

This review summarizes current data on modification of primary structure of proteins resulting from their direct interaction with reactive species of oxygen, nitrogen, and sulfur, on interaction of products of free radical oxidation of lipids with proteins and its functional consequences at the level of cells, tissues, and whole organisms. Some attention also will be paid to proteins that change their functional role due to oxidation, for example, cytosolic form of aconitase, which under oxidation can be converted into a protein regulating iron metabolism.

CHEMICAL MODIFICATION OF POLYPEPTIDE CHAINS

The chemical modification of polypeptide chains and non-protein components should be distinguished on the interaction of reactive species with complex proteins. The modification of the polypeptide part may affect peptide bond, as well as side chains of amino acid residues. As for modification of non-protein part of complex proteins, such modification is best studied on proteins containing non-heme iron, as a component of Fe,S-clusters. Under oxidative conditions iron ions are released from the proteins, which results in loss of enzymatic activity. This group of proteins includes, for example, some enzymes of the Krebs cycle, namely, aconitase, succinate-, isocitrate-, and malate dehydrogenases, and fumarase [5-7]. The detailed mechanisms and functional consequences of these changes will be analyzed below. Further we will focus on free radical modification of polypeptides, particularly on interaction of free radicals with peptide bond and side chains of amino acid residues [8, 9].

Attack on and cleavage of peptide bonds. Figure 1 demonstrates the suggested pathways of oxidation of components forming a peptide bond [8-11]. It is seen that the abstraction of hydrogen atoms by hydroxyl radical from the α-carbon atom of any amino acid residue begins a sequence of reactions resulting in the formation of alkyl radical and water (*a*). The subsequent addition of an oxygen molecule to the alkyl radical gives an alkylperoxy radical (*b*), reacting with protonated superoxide anion ($HO_2^{\cdot}$) or Fe^{2+} and H^+ (*c*). The formed alkylperoxide again may enter reactions with alkoxyl radical [2]. The peptide bond can be cleaved at this stage, or the abovementioned radical can be further oxidized by protonated superoxide or Fe^{2+} and H^+ with the formation of hydroxyl derivative of a peptide (*d*). It should be noted that alkyl, peroxyl, and alkoxyl derivatives of peptides also may abstract hydrogen atoms from amino acid residues, giving rise to new radical species, which are able to enter into similar transformations. In the absence of oxygen or at its low concentrations, two alkyl derivatives

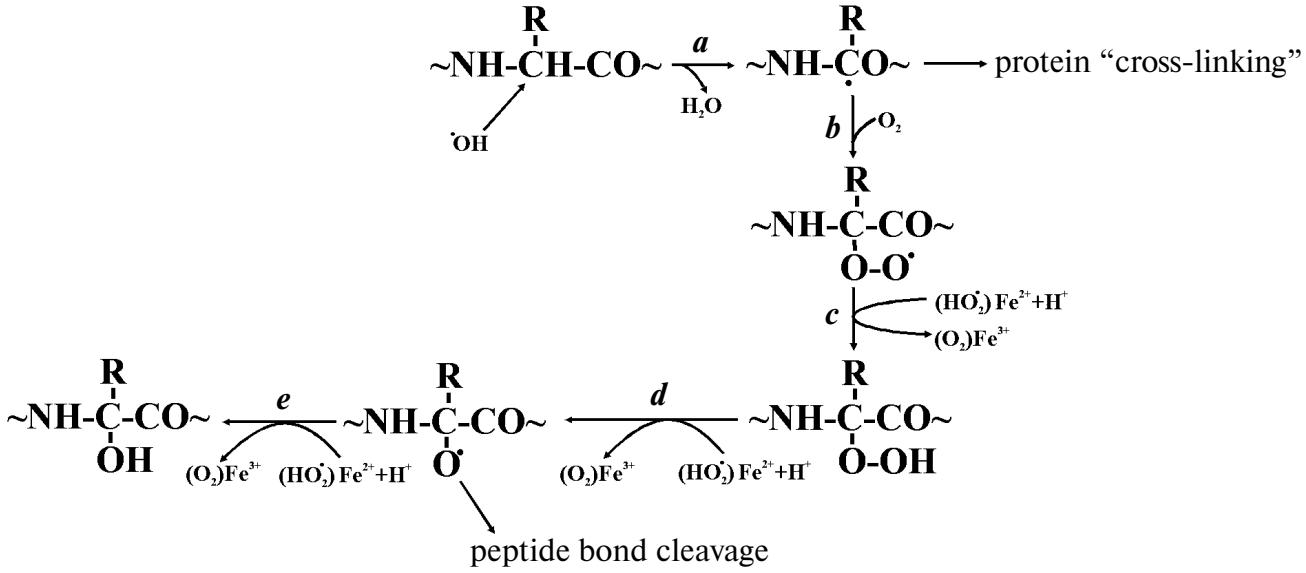

Fig. 1. Possible pathways of oxidation of peptide chain (modified from [8-11]).

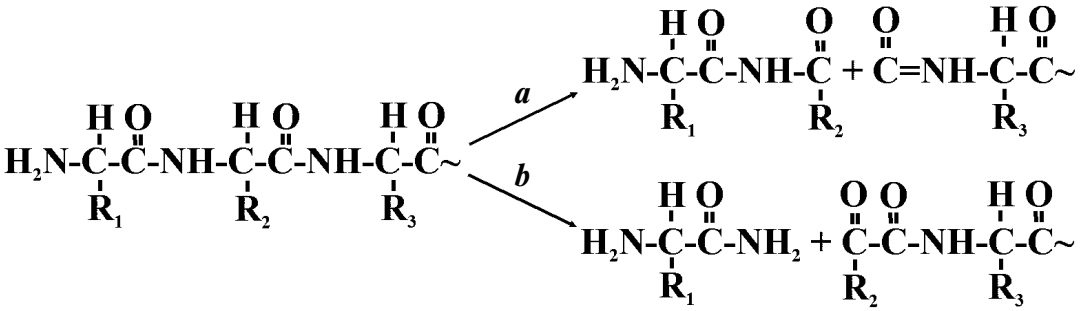

Fig. 2. Possible pathways leading to peptide bond cleavage (modified from [8-11]).

of polypeptides may combine forming intra- and/or interpeptide links.

Up to now at least four mechanisms of free radical-induced peptide bond cleavage have been proposed: (*a*) cleavage of alkoxyl derivative peptides via the α-amide pathway, (*b*) cleavage of alkoxyl derivative peptides via the diamide pathway, (*c*) oxidation of side chains of glutamyl and aspartyl residues, and, finally, (*d*) oxidation of side chains of proline [8, 9]. Figure 2 schematically depictures mechanisms (*a*) and (*b*). The N-terminal amino acid residue of the peptide fragment obtained from the C-terminal portion of the protein exists as an isocyanate derivative, whereas the C-terminal portion of the protein derived from the N-terminal portion exists as a diamide derivative (*a*). In pathway (*b*), the N-terminal amino acid residue of the peptide derived from the C-terminal portion exists as an α-ketoacyl derivative, and the C-terminal amino acid residue of the other peptide exists as an amide derivative.

Specific cleavage of a peptide bond induced by oxidation can take place at monoamino-dicarboxylic residues. Figure 3 shows a possible mechanism of peptide bond cleavage by the glutamate oxidation pathway (similarly for aspartate) (route (*c*)). This pathway is initiated by ˙OH-dependent abstraction of a hydrogen atom from the γ-carbon of the glutamyl side chain, which is followed by a series of reactions analogous to those given in Fig. 1. These reactions result in peptide bond cleavage and the formation of oxalic acid. The peptide fragments produced are represented by amide from the former N-end and by blocked pyruvyl moiety from the former C-terminal portion.

Schuessler and Schilling found that the number of peptides formed during the exposure of proteins to ionizing radiation was approximately equal to the number of prolyl residues present (cited after [10]). Further it was demonstrated [11] that the oxidation of prolyl residues leads to the formation of the 2-pyrrolidine derivative and peptide bond cleavage according to the reaction given in Fig. 4. Upon acid hydrolysis, 2-pyrrolidone is converted to 4-aminobutyric acid. Therefore, the presence of 4-aminobutyric acid in acid hydrolyzates of proteins is presumptive evidence for peptide cleavage by the 2-pyrrolidone pathway.

Another important aspect of free radical processes in biological systems should be noted. Iron ions play a critical role in free radical oxidation of proteins. They can be involved in the initiation of these processes by donating electrons. Because iron ions are easy to oxidize and reduce and the cell contains relatively high iron concentrations, their role in oxidative damage of biological structures, particularly proteins, is very important. However, in fact, there are no free iron ions in the cell or their concentrations are negligible because cells possess special systems for iron sequestration. However, under certain pathological conditions iron ions may be released from cellular depots, which may intensify free radical processes with the following consequences.

Oxidation of side chains of amino acid residues. In fact, all amino acid residues of peptides are susceptible to oxidation by hydroxyl radicals. However, the formed products are characterized only in a few cases. The table summarizes established products of oxidation of protein amino acid residues. To simplify the analysis of the introduced modifications in side amino acid chains they may be grouped as: aromatic amino acids, amino acids oxidation of which gives carboxyl groups, and sulfur-containing amino acids.

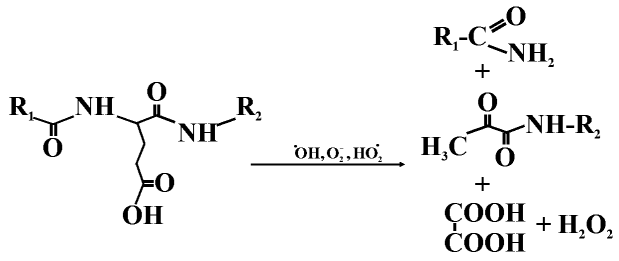

Fig. 3. Possible mechanisms of peptide bond cleavage at glutamic acid residues. Similarly, the peptide can be cleaved at aspartate residues (modified from [8-11]).

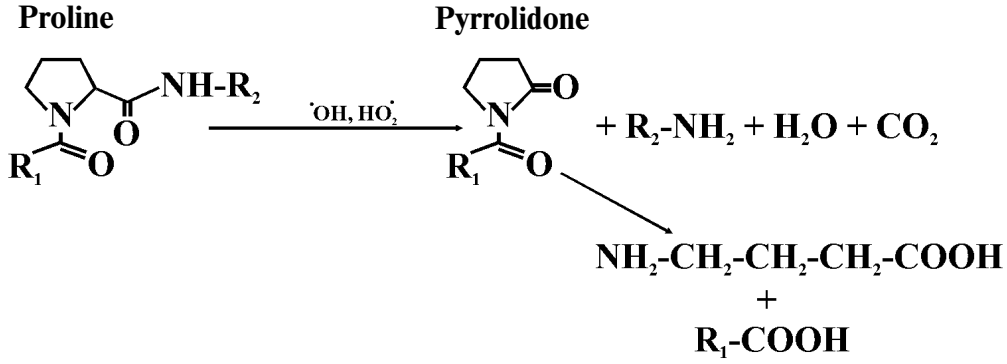

Fig. 4. Hypothetical pathways of oxidative cleavage of peptide bonds at proline residues. The stable product 4-aminobutyric acid is formed as the end product (modified from [8-11]).

Aromatic amino acid residues are particularly susceptible to oxidation by ROS. Under these conditions, phenylalanine is converted to both mono- and dihydroxy derivatives, and tyrosine is converted to the 3,4-dihydroxy derivative. The formed compounds can undergo redox cycling, and due to it they can be involved in ROS production. Tyrosyl radicals, formed on oxidation of tyrosine, can interact with another one to form intra- and/or interprotein cross-linkages. Therefore, the presence of such 2,2′-biphenyl derivatives can serve as a marker of ROS-induced oxidative damage of proteins [12]. The attack of the tyrosine residues by reactive nitrogen and chlorine species results in the formation of nitro- and chloro-derivatives. Tryptophan is highly sensitive to oxidation by γ-radiation, which leads to various hydroxy derivatives, formyl-kynurenine and 3-hydroxy-kynurenine. Under ultraviolet irradiation in the presence of ozone, high levels of Fe^{2+} and H_2O_2 or peroxynitrite, tryptophan can be converted to kynurenine and N-formyl-kynurenine. It was also found that tyrosine and tryptophan residues are not among major targets for oxidation in systems with physiological concentrations of transition metal ions, presumably because these residues usually are not present at metal binding sites of proteins. In contrast to tyrosine and tryptophan residues, histidine, arginines, and lysine residues are highly sensitive targets for free radical induced oxidation in systems with physiological concentrations of transition metal ions. It is supposed this is due to their localization in metal binding sites of proteins, and moreover these residues are often involved in the formation of coordinate bonds between proteins and ions [8, 9].

Identified products of free radical oxidation of amino acid residues of proteins [10, 11]

Residue	Products
Phenylalanine	2,3-dihydroxyphenylalanine, 2-, 3-, and 4-hydroxyphenylalanine
Tyrosine	3,4-dihydroxyphenylalanine, 3-nitrotyrosine, chlorotyrosine, dityrosine (2,2'-biphenyl derivatives)
Tryptophan	kynurenine, 3-hydroxy-kynurenine, oxindole, hydropyrroloindole, N-formyl-kynurenine
Histidine	2-oxohistidine, 4-OH-glutamate, aspartate, asparagine
Lysine	2-aminoadipic semialdehyde
Arginine	glutamic semialdehyde
Proline	glutamic semialdehyde
Threonine	2-amino-3-ketobutyric acid
Glutamic acid	pyruvic acid
Aspartic acid	pyruvic acid
Cysteine	nitrosothiols, thiol radicals, cystine, conjugates with glutathione
Methionine	methionine sulfoxide, methionine sulfone

The oxidation of some amino acid residues leads to carbonyl derivatives, which has a special interest. The table shows that groups mentioned may be formed on oxidation of the side chains of lysine, arginine, histidine, proline, threonine, glutamic acid, and aspartic acid. The oxidation of the first four amino acid residues converts them directly into aldehyde or ketone derivatives, while the oxidation of two last results in peptide bond cleavage

reaction in which the N-terminal amino acid of one peptide fragment is blocked by a pyruvyl moiety (Fig. 2). Oxidative cleavage of the polypeptide chain by the α-amidation pathway leads to the formation of a peptide in which the N-terminal amino acid is blocked by a 2-ketoacyl derivative. In addition, the reactions of proteins with reducing carbohydrates and/or their oxidation products (glycation and glycoxidation reactions) or with lipid peroxidation products (malondialdehyde, 2- and 3-unsaturated aldehydes, in particular 4-hydroxy-2-nonenal) also may lead to the introduction of new carbonyl groups in proteins. The formation of additional carbonyl groups in proteins as a result of their oxidative modification is used most frequently to evaluate the intensity of this process with dinitrophenylhydrazine [13, 14]. The hydrazines formed are easily measured by spectroscopic techniques and are considered as a reliable marker of protein modification induced by free radicals.

Cysteine and methionine residues in proteins are particularly susceptible to oxidation by in fact all forms of reactive species of oxygen, nitrogen, sulfur, and chlorine. But, in contrast to other amino acid residues, the partially oxidized forms of cysteine and methionine can be reduced by special enzymatic systems. Particularly these species undergoing reversible oxidation are frequently used by the cell either for regulation of some cellular processes or as antioxidants. The oxidation of cysteine residues results in the formation of sulfenic, sulfinic, and sulfonic acid derivatives [10]. Sulfenic derivatives may be either further oxidized to sulfinic derivatives, or to form mixed ethers with cysteine or glutathione, or reduced to cysteine via nonenzymatic (in experiment by dithiothreitol treatment) or by enzymatic mechanisms with the involvement in later case of glutaredoxin, a glutathione-specific thiol transferase. It is supposed that the formation of mixed ethers of sulfenic acid with glutathione may prevent following irreversible sulfur oxidation [10]. Some examples of cysteine reactions residue are given in Eqs. (1)-(5) [10].

$$R_1SH + R_2SH + H_2O_2 \rightarrow R_1\text{-}S\text{-}S\text{-}R_2 + 2H_2O, \quad (1)$$

$$R_1\text{-}S\text{-}S\text{-}R_2 + GSH \rightarrow R_1SH + GS\text{-}SR_2, \quad (2)$$

$$GS\text{-}SR_2 + GSH \rightarrow R_2SH + GS\text{-}SG, \quad (3)$$

$$GS\text{-}SG + NADPH + H^+ \rightarrow 2GSH + NADP^+, \quad (4)$$

$$\text{Glucose-6-phosphate} + NADP^+ \rightarrow$$

$$\rightarrow \text{6-phosphoglucolactone} + NADPH + H^+. \quad (5)$$

The formed disulfide derivative (reaction (1)) undergoes glutathione–disulfide exchange (reactions (2) and (3)), leading to the formation of the initial cysteine-containing peptides. Water and oxidized glutathione are the net products of reactions (1)-(3). In order to continue the operation of the system it should be "recharged", i.e. it is necessary to restore reduced glutathione resources. Glutathione reductase regenerates reduced glutathione at the expense of NADPH reducing equivalents (reaction (4)). Several reactions can provide NADPH, and glucose-6-phosphate dehydrogenase is commonly the main supplier (reaction (5)), but isocitrate dehydrogenase, transhydrogenase, and malate dehydrogenase also catalyze $NADP^+$ reduction. So, we have analyzed one more link between metabolism of reactive oxygen species and carbohydrates.

Methionine residues also can undergo oxidation with the formation of methionine sulfoxide (MeSOX) and methionine sulfone. Methionine sulfoxide can be reduced to methionine [15]:

$$\text{methionine} + H_2O_2 \rightarrow MeSOX + H_2O, \quad (6)$$

$$MeSOX + T(SH)_2 \rightarrow \text{methionine} + T(S)_2, \quad (7)$$

$$T(S)_2 + NADPH + H^+ \rightarrow T(SH)_2 + NADP^+. \quad (8)$$

The oxidation of methionine results in MeSOX formation (reaction (6)). Methionine residues can be oxidized not only by H_2O_2, but also many other oxidants. Methionine sulfoxide can be reduced to methionine by methionine sulfoxide reductase (reaction (7)). Thioredoxin ($T(SH)_2$) serves as an electron donor for this reaction. The later is regenerated at the expense of NADPH in the reaction catalyzed by thioredoxin reductase (reaction (8)).

It is interesting to note that residues of sulfur-containing amino acids are readily oxidized, but at the same time can be also reduced to their initial forms. The described properties of residues of sulfur-containing amino acids unable their use as "incorporated" protein antioxidants. Moreover, methionine residues are rarely involved in catalytic process because most methionine residues are localized outside the active site of enzymes. Therefore, methionine residue oxidation usually either does not affect biological protein functions, or affect negligibly. However, in many cases a cysteine residue is involved in catalytic process. For example, in protein tyrosine phosphatase, oxidation leads to the inactivation of the enzyme [16]. It should be added that oxidation of methionine residues makes them more hydrophobic and they became more susceptible to degradation by multicatalytic protease [17, 18].

Free radical nitric oxide ($^{\cdot}NO$) is an important metabolite of arginine catabolism. It regulates many cellular processes. At the same time, being a highly active compound, it undergoes many chemical reactions. Nitric oxide reacts with superoxide anion forming the highly toxic peroxynitrite $OONO^-$ [1]. The later is an extremely reactive compound. Reactions of peroxynitrite

and hydroxyl radical with tyrosine residues in proteins lead to their nitration and oxidation with the formation of tyrosine–tyrosine cross-linkages, 5-nitrotyrosine, dihydroxyphenylalanine, and oxidation of tryptophan, methionine, and cysteine residues. The modification of tyrosine residues needs special attention because in regulatory proteins they are reversibly phosphorylated by tyrosine protein kinases and phosphatases. Therefore, their nitration and oxidation may block phosphorylation by suitable kinases. Contrary to phosphorylation, nitration and oxidation are usually irreversible processes. Therefore, this class of the enzymes is removed from regulatory pathways.

Finally, we will analyze information on oxidation-induced formation of protein–protein cross-linkages. In the literature, one can find at least six different pathways that can result in the formation of inter-protein cross-linkages [8-11].

1. Interaction of two alkyl radical protein derivatives formed on oxidation of protein backbone or amino acid side chains:

$$P^1R_1C^{\cdot} + P^2R_2C^{\cdot} \rightarrow P^1R_1CCR_2P^2 . \quad (9)$$

2. Interaction of two tyrosyl radicals:

$$P^1tyr^{\cdot} + P^2tyr^{\cdot} \rightarrow P^1\text{-}tyr\text{-}tyr\text{-}P^2 . \quad (10)$$

3. Interaction between malondialdehyde and other dialdehydes with amino groups of lysine residues in two different protein molecules:

$$P^1\text{-}NH_2 + P^2\text{-}NH_2 + CH_2(CHO)_2 \rightarrow$$

$$\rightarrow P^1\text{-}N{=}CHCH_2CH{=}N\text{-}P^2 + 2H_2O. \quad (11)$$

4. Interaction of products of Michael addition (product of interaction between 4-hydroxy-2-nonenal (HNE) with any protein) with amino group of lysine residue of a second protein molecule:

$$P^1\text{-}HNE\text{-}CHO + P^2\text{-}NH_2 \rightarrow$$

$$\rightarrow P^1\text{-}HNE\text{-}CH{=}NP^2 + H_2O . \quad (12)$$

5. Interaction of carbonyl group of products of glycation of one protein with lysine amino group of a second protein:

$$P^1R_1CHO + P^2\text{-}NH_2 \rightarrow$$

$$\rightarrow P^1R_1\text{-}CH{=}N\text{-}P^2 + H_2O. \quad (13)$$

6. Oxidation by cysteine residue reactive species (RS) of two different proteins:

$$P^1SH + P^2SH + RS \rightarrow$$

$$\rightarrow P^1S\text{-}SP^2 + \text{reduced RS}. \quad (14)$$

The consequences of the formation of cross-linked protein complexes are studied insufficiently. For example, it is known that cross-linked proteins are not degraded by multicatalytic proteases and/or proteasomes, which may result in accumulation of oxidized proteins during aging and in some pathologies [8-11]. Moreover, these complexes can inhibit proteolytic degradation of other oxidized proteins.

Another interesting consequence of free radical-induced protein oxidation should be mentioned. Using the method of hydrophobic chromatography on a phenyl matrix, it was found that oxidation of glutamine synthase from *E. coli* first resulted in the formation of a more hydrophilic than initially enzyme form, which was associated with inactivation of the enzyme [19, 20]. The more hydrophilic form was not hydrolyzed by purified specific protease from *E. coli*. Further oxidation formed a more hydrophobic form, which was hydrolyzed by the above-mentioned protease specific to the oxidized form of glutamine synthase [19]. Base on these results, one can suggest that partial oxidation of proteins, modifying surface charge or hydrophobicity, is one of the mechanisms determining their spatial distribution in the cell [21, 22].

MODIFICATION OF PROTEINS BY PRODUCTS OF FREE RADICAL OXIDATION OF LIPIDS

Interaction of lipids with ROS gives a large array of oxidized products, and malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are most known. Although the first has received enough attention, the role of the later has been investigated only during the last decade. MDA effects are connected with two aldehyde groups in its molecule, which make possible its interaction with amino groups of proteins and because of bifunctionality make cross-links forming Schiff bases. HNE is an α,β-unsaturated aldehyde. It is supposed that cytotoxic effects of HNE are connected with at least four processes: 1) inactivation of enzymes; 2) erythrocyte lysis; 3) chemotoxicity to neutrophils; and 4) inhibition of protein and DNA synthesis [23, 24].

The interaction of HNE with free amino acids and their residues in proteins has been called Michael addition (reaction (12)) (Fig. 5). The character of formed products depends on the type of amino acid. For example, it is supposed that the interaction of HNE and histidine results in a cyclic product, which is rather stable and can be found in proteins in biological systems subjected to oxidative stress [25]. The formed cyclic products of interaction between HNE and lysine or cysteine may further give carbonyl groups. Secondary reactions of products of

Michael addition with amino groups of lysine residues of proteins can lead to the formation of intra- and intersubunit cross-links [26].

Highly sensitive chemical [23, 25, 26] and immunochemical [25-28] methods for identification of products of interaction between HNE and proteins have been developed. Polyclonal antibodies to HNE-modified hemocyanin were developed by immunization of rabbits [25, 28]. The recognized epitope is 2-$CH_3(CH_2)_4$-5-hydroxy tetrahydrofuran [28]. In atherosclerotic plaques of human aorta, these antibodies strongly interacted with granular cytoplasmic elements of foam cells, but weakly interacted with surrounding sclerotic stroma [28]. Using polyclonal antibodies, specifically modified proteins were found in hepatocytes treated with HNE, or subjected to *tert*-butylhydroperoxide, mixture of ascorbate and iron ions, or by iron ions with 15-hydroperoxyeicosatetraenoic acid [25]. Incubation of enzymes with HNE inactivated them. For example, the treatment of glyceraldehyde-3-phosphate dehydrogenase (GAPD) from *Leuconostoc mesenteroides* with HNE inactivated the enzyme in time- and concentration-dependent manner [29]. The inactivation was accompanied by increase in protein carbonyl concentrations. The treatment of GAPD with 2 mM HNE at 36°C during 12 h resulted in modification of 74% of cysteine, 16% of lysine, and 50% of histidine residues. Glyceraldehyde-3-phosphate, a substrate of the enzyme, did not affect the modification of either cysteine or histidine residues, but protected one histidine residue per enzyme monomer. The coenzyme NAD^+ did not affect the modification of lysine residues but protected one cysteine and two histidine residues [26].

In experiments with ischemia/reperfusion of rat heart, the possible involvement of HNE in inactivation of mitochondrial enzyme cytochrome oxidase was found [30]. A 15-min ischemia followed by reperfusion significantly reduced the enzyme activity and was associated with increased HNE concentration in mitochondria. Enhanced concentration of HNE–cytochrome *c* oxidase adducts were found in ischemic/reperfused rat heart homogenates by the Western blotting method. The data with heart tissue were confirmed *in vitro* with the use of purified enzyme, treated with HNE, and reduced glutathione prevented the enzyme inactivation. Therefore, it becomes clear that interaction of HNE, similarly to other products of free radical lipid oxidation, can result in the formation of respective products. Under these conditions, many enzymes loose their activity. The inactivation of GAPD or other glycolytic enzymes can lead to inhibition of glycolysis and is followed by reduced cell energy supply. If products of free radical lipid oxidation modify components of the electron-transport chain, in addition to decreased ATP production, it may increase ROS generation. The later may seriously worsen cell state up to its death.

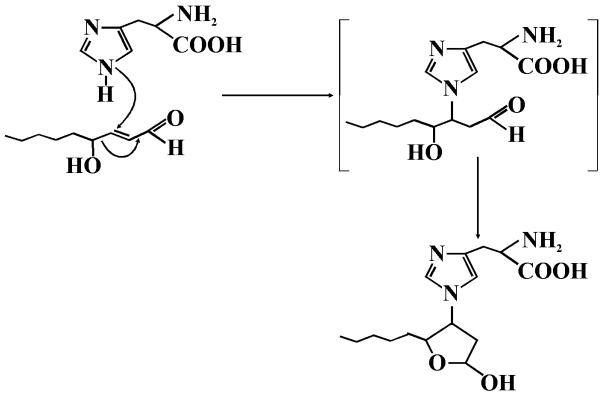

Fig. 5. Interaction of 4-hydroxy-2-nonenal (HNE) with free histidine. This reaction between HNE and free or protein-incorporated amino acids is called Michael addition (modified from [8]).

EFFECT OF OXIDATIVE MODIFICATION ON ENZYME PROPERTIES

The inactivation or modification of regulatory properties of enzymes frequently results from enzyme modification by reactive species. The final result of the effects of reactive species on enzymes depends on the nature of both participants and the presence of substrates, products, inhibitors, or activators. In order to introduce the reader to the chemical principles and possible consequences of interaction between proteins/enzymes and reactive species, we will describe a well-studied system, which was first used to elucidate the main basic principles. We mean glutamate synthase (GS) from enterobacteria *Escherichia coli*. This enzyme has been studied for more than 20 years in laboratories of Drs. E. Stadtman and R. Levine with colleagues (National Institutes of Health, USA) and it creates a good basis for systematic analysis of effects of free radicals on enzymes [31]. In the early 1980s, it was found that intracellular GS degradation was carried out in two phases [32-37]. In the first stage, oxidative modification inactivates the enzyme. Further, the oxidized GS was hydrolyzed by specific protease [38]. The presence of substrate and level of the enzyme adenylation affected oxidation intensity, which may provide fine control of active molecules. In a model *in vitro* system, the oxidation of one of 16 histidine residues in the monomer resulted in GS inactivation. One additional carbonyl group was found. The amount of other oxidizable amino acids, namely cysteine, methionine, phenylalanine, tyrosine, and tryptophan was not changed. To perform oxidative modification, hydrogen peroxide, ascorbate, and dithiothreitol were used with iron ions [34]. Inactivation of GS in a system generating superoxide anion was stimulated by proteins containing

non-heme Fe,S-clusters and was partially delayed by antioxidants, namely superoxide dismutase, histidine, mannitol, and dimethylsulfoxide, and it was fully blocked by Mn(II), EDTA, and catalase [35].

Glutamine synthase contains a cation-binding site. It was found that this site is a target for attack by ROS [36]. Up to certain concentrations of ions with changeable valence, free radical-induced oxidation results in the formation of peptides with reproducible structure. The formed fragments contained the Met-His-Cys-His-Met sequence, in which just the histidine residues were oxidized [37]. Although the oxidation of one histidine residue resulted in inactivation of the enzyme, the second histidine residue oxidation was necessary for the enzyme to be recognized by specific protease [38, 39]. Oxidation of the protein changes its physicochemical properties. A more hydrophilic protein is formed at the first oxidation stage [19]. This form is not a protease substrate. However, further oxidation forms a more hydrophobic protein than the initial and this form is proteolytically degraded [36]. Oxidation of GS reduced its thermostability and isoelectric point, character of inter-subunit interactions, which was associated with the loss of histidine residues, and appearance of new carbonyl groups [40]. Under these conditions, one histidine residue (His269) was converted into asparagine and an arginine residue (Arg344) into γ-glutamyl-semialdehyde [41]. Oxidation also changed the structure of the active center and slightly decreased the strength of inter-subunit interactions and molecular packing [42]. The latter two changes were supposed to be responsible for reduction of the thermal stability of the enzyme.

Glutamine synthase can be inactivated not only by ROS. Treatment of the enzyme with peroxynitrite resulted in nitration of tyrosine residues and transformation of methionine into methionine sulfoxide [43]. The Fe-EDTA complex stimulated only the first process. Only one tyrosine residue per subunit of non-adenylated GS was nitrated without stimulation, which modified the enzyme requirements for bivalent cations, pH sensitivity, affinity to ADP, and sensitivity to inhibition by pathway end-products—tryptophan, AMP, and CTP. However, nitration of the second tyrosine residue per subunit of adenylated GS led to full inactivation of the enzyme. In the presence of Fe-EDTA, nitration has more variable character—the modification of 5-6 tyrosine residues per subunit imitated the transformation in non-adenylated GS form. Therefore, the authors concluded that the nitration, being an irreversible process, might seriously affect cascades of signal transduction where reversible phosphorylation and adenylation are involved [43]. It should be noted that nitration can be regulated not only by substrates. For example, carbon dioxide can accelerate nitration of tyrosine residues [44].

The works described above suggest the following scheme of involvement of reactive oxygen and nitrogen species in GS modification. Under high ammonium level in media, GS operates actively involving ammonium into cellular metabolism. Although the intensity of metabolism is rather high, which is accompanied by ROS production, the presence of ammonium protects GS from inactivation. Under conditions of either low ammonium concentration or its absence, there is no need for GS. Therefore, the degradation mechanism is switched on, which is triggered by free radical-induced oxidation. The absence of ammonium makes the process easier, which increases its rate. The binding of iron ions with specific GS sites also stimulates the oxidation. At the first stage the enzyme is inactivated, but not degraded by protease. It seems this stage is preparative to the next one, which induces conformation change of GS and makes it a substrate of a specific protease.

Information about the oxidation of methionine residues of GS was mentioned above. Although the specific function for methionine residues in proteins is unknown, they can be reversibly oxidized [19, 20]. GS from *E. coli* contains 16 methionine residues per subunit, and oxidation of eight of them in fact does not influence its activity. Since oxidizable residues are localized at the surface of the molecule, it was natural to suggest their protective role [20]. It also should be added that alkyl peroxides and alkylperoxy radicals modify GS from *E. coli* [45]. Treatment by the abovementioned compounds fully inactivated the enzyme, which was accompanied by the loss of histidine, tyrosine, methionine, and tryptophan residues, polypeptide fragmentation, and formation of high molecular mass aggregates.

There is considerable information on oxidative inactivation of enzymes from different organisms. These include acetyl-CoA hydrolase, acetylcholine esterase, alkaline phosphatase, alcohol dehydrogenase, catalase, fructose-1,6-biphosphatase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, and many other enzymes. These works have been analyzed in an excellent review [46]. However, since the time of its publication many new works in this direction have appeared. For example, at oxidative modification of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, it was found that incubation with hydrogen peroxide and Fe^{2+} quickly inactivated the enzyme. This process was accompanied by an increase in heat-induced inactivation [47, 48].

Lactate dehydrogenase, being relatively resistant to oxidation, can also be inactivated by ROS [49]. We compared two isoforms—from bovine heart (H_4) and skeletal (M_4) muscles (unpublished data). The enzyme from metabolically more active heart muscle demonstrated higher tolerance to oxidative inactivation than the enzyme from skeletal muscle.

The inactivation of enzymes by ROS due to interaction of the latter with the polypeptide part was analyzed above. The activity of many enzymes also depends on the

presence of non-peptide components. The enzymes containing so-called (4Fe-4S) clusters, for example dehydratases, are interesting from this point of view. The release of iron ions from their active sites may result in their oxidative inactivation. Dehydratases of α,β-dihydroxy acids and 6-phosphogluconate as well as aconitase contain this cluster. In *E. coli* O_2^- inactivated α,β-dihydroxyisovalerate dehydrogenase both *in vitro* and *in vivo* [5]. Fumarase is also sensitive to O_2^-. Under oxidative stress conditions the constitutive form of this enzyme is replaced by another molecular form that does not contain Fe,S-cluster [50]. Using *E. coli*, it was also found that one more enzyme containing [4Fe-4S] cluster, aconitase, was inactivated by O_2^- [6]. Such sensitivity of Krebs cycle enzymes to ROS may be useful for the cell. Reducing equivalents, NAD(P)H and $FADH_2$, formed during operation of the cycle are used for ATP production, but some of the reducing equivalents may be used for one electron reduction of molecular oxygen to superoxide, which may damage cellular components. There are two potential ways to prevent negative effects: either using of antioxidants or by inactivation of complex I of the electron transport chain. Both possibilities can be used also simultaneously. The ROS-induced inactivation of proteins may be a reason for reduced survival of *E. coli* strains lacking superoxide dismutase or catalase [51-53]. In our works on baker's yeast *Saccharomyces cerevisiae*, a strong negative relationship between activities of catalase and superoxide dismutase and sensitivity to ROS was found, and this will be analyzed below.

Studies on eukaryotic aconitase demonstrated another interesting aspect of the role of ROS in the cell. Its mitochondrial form along with α-ketoglutarate and succinate dehydrogenase is very sensitive to oxidation [54]. In addition to the mitochondrial form, eukaryotic cells possess a cytosolic form. Its [4Fe-4S] cluster can be oxidized with the loss of enzymatic activity, and the resulting [3Fe-4S] form is able to bind mRNA, coding proteins that regulate iron metabolism [55, 56]. Binding to 3′-untranslated end of transferrin mRNA blocks availability for RNase, increases its half-life, and it finally increases transferrin synthesis. In addition, the [3Fe-4S] form can bind the 5′-untranslated end of ferritin mRNA. This blocks the formation of pre-initiation complex and reduces transcription intensity. It should be added that [4Fe-4S] → [3Fe-4S] transitions are reversible and are regulated not only via oxidation/reduction processes, but also by intracellular iron levels [56, 57].

Since mitochondria produce over 90% of ROS in eukaryotic cells, it is easy to understand the interest in oxidative modification of mitochondrial enzymes, particularly α-ketoglutarate and glutamate dehydrogenases. α-Ketoglutarate dehydrogenase contains residue of lipoic acid needed for enzymatic activity. The mentioned residue is a strong hydrophobic nucleophile, which makes it highly sensitive target for hydrophobic electrophile. HNE is one of candidates that can attack lipoate. In fact, *in vitro* HNE inactivated α-ketoglutarate dehydrogenase [58, 59]. The enzyme was inactivated in isolated mitochondria by hydrogen peroxide [54] and during ischemia/reperfusion of isolated rat hearts [57]. Complexes I and IV of the electron transport chain are also inactivated on oxidation by ROS [54, 57]. As mentioned above, inactivation of cycle Krebs enzymes at increased ROS levels may be useful for the cell because it reduces intensity of formation of reducing equivalents, NAD(P)H and $FADH_2$, part or which can be used to generate ROS. The situation with components of the electron transport chain is more complicated. The inactivation of complex IV may increase ROS production by electron carriers, and inactivation of complex I may partially reduce the risk, decreasing the number of electrons entering the electron transport chain. Therefore, the final result depends on many factors, and inactivation of Krebs cycle enzymes as well as carriers of the electron transport chain may play both negative and positive roles.

Bunik and colleagues found that dehydrogenases of 2-oxo acids (OXDH) also are sensitive to ROS [58, 59]. During the catalytic process catalyzed by OXDH, lipoate undergoes reversible oxidation, which is reduced by thioredoxin. FAD, one of the components of the complex, is responsible for ROS generation, particularly for O_2^- production, which results in formation of thiyl radical of lipoate. These events inactivated the enzyme. Superoxide dismutase did not prevent the enzyme inactivation, but thioredoxin, a scavenger of thiyl radicals, was a protector. Therefore it was concluded that thiyl radical of bound lipoate induced the inactivation via one electron oxidation of OXDH catalytic intermediate. The authors supposed that the prooxidant effect of dehydrolipoate, bound in complex, is controlled by thioredoxin and cellular pool of NAD^+ and NADH [58, 59]. It follows that under certain conditions oxidoreductases can also generate reactive species. In the above analyzed case, they probably produce superoxide anion and thiyl radical. However, to reduce the chance to be damaged, cells possess efficient protective systems, particularly the special protein thioredoxin. The later, being dependent on the level of "charge" of the NAD^+/NADH system, can protect dehydrogenases as well as other enzymes, providing a negative feedback regulatory system.

PROTEIN OXIDATION AS A MARKER OF AN ORGANISM'S STATE

In recent years, the concentration of oxidized proteins has been widely used to evaluate the intensity of oxidative stress *in vivo*, i.e. the state when steady-state concentrations of ROS are enhanced. Since absolutely all organisms possess proteins, their oxidation may be a reliable marker of the intensity of oxidative processes. There

are several reasons why measurement of oxidized proteins is preferred over oxidized lipids and nucleic acids. Since proteins carry out specific biological functions, it is possible to register not only the formation of oxidation products, but also modification of protein function. The end products of protein oxidation are rather stable, and many highly sensitive methods for analysis of products of free radical oxidation of proteins can give some clues to recognize the type of oxidants involved in an oxidative process [60].

Both the above-listed and some other factors have led to broad use of concentration of oxidized proteins as markers of oxidative stress intensity. The measurement of additionally formed carbonyl groups, one of the stable products of protein oxidation, is the most frequently used marker. For example, a transient increase in serum protein carbonyls was found in heart surgery [61]. An increase in protein carbonyl groups in tissues of the frog *Rana ridibunda* accompanied recovery of the animals from winter anabiosis [62]. Therefore, further we will describe some states of organisms where the activation of free radical processes is reliably seen.

Aging. During aging of an organism, oxidized proteins are accumulated, particularly so-called "aging pigments" containing proteins, for example, lipofuscin [63-68]. The question "What is the primary process, protein oxidation or aging?" is critical in this particular case as well as at some pathological states. As mentioned above, the character of oxidized products depends substantially on the type of involved reactive species. Since additional carbonyl groups in proteins are formed on oxidation by virtually any ROS form, they are most frequently used as a marker.

In works of Professor R. Sohal's laboratory not only the accumulation of carbonyl groups in proteins (CP) during aging, but also the relationship between their concentration and life duration were studied. In the housefly the concentrations of protein carbonyl groups were more tightly bound to physiological age than to chronological age [69, 70]. Reduction of the physiological activity of flies extended not only mean, but also maximal life duration. These data are well related with the known fact of increased ROS generation by mitochondria of aged flies. Since increased ROS production by mitochondria of aged flies was closely related to accumulation of protein carbonyls in this organelle, it was concluded that the accumulation of oxidized proteins is responsible for the investigated processes [71]. In the same laboratory another model, gerbil, was used for aging studies. During aging the concentrations of protein carbonyls were increased in all brain parts, and a similar effect was found in heart and brain cortex of animals [72]. Introduction of the spin trap with antioxidant properties α-phenyl-N-*tert*-butylnitrone (PBN) decreased the concentrations of protein carbonyl groups in gerbil brain cortex [72], confirming earlier experiments [73]. PBN also extended the life duration of the housefly. Therefore, the authors concluded that although antioxidants can protect tissues from oxidative modification, their effects are both tissue and species specific. It was elucidated that changes in cognitive and motor functions in mice were also associated with age-dependent accumulation of protein carbonyls in the brain. The capacity for learning was positively related with concentration of protein carbonyls in brain cortex, while reduction of motor coordination—with protein oxidation in cerebellum [74].

The aging of human red blood cells is accompanied not only by the accumulation of protein carbonyl groups, but also by decrease in activities of certain enzymes, namely glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and aspartate aminotransferase [75]. In fibroblast cells of healthy humans, the concentrations of protein carbonyls were higher only for individuals older than 60 years of age. However, in fibroblasts from humans with certain pathologies, such as progeria or Werner's syndrome, the concentrations of protein carbonyls was two-fold higher before age 60 than that in healthy persons [75]. The accumulation of oxidized proteins takes place in many organs. For example, the amount of highly oxidized carbamyl hydrase III in rats increased with aging. This enzyme possesses three known activities—carboanhydrase, esterase, and phosphatase. While during aging the first two activities reduced by approximately 30%, the third activity disappeared completely [76]. Earlier we wrote that the accumulation of protein carbonyls can be associated with ROS generation by mitochondria. But similar results can also be due to decrease in activity of proteases, particularly those degrading oxidized proteins. In liver and other rat tissues, a multicatalytic protease, discriminating native and oxidized substrates, was found. In different experiments, carried out in the same laboratory using the same rat lines, different results were obtained—either significant decrease during animal aging or no changes [77, 78]. It is clear that in experiments carried out on living animals it is impossible to account for all factors affecting free radical processes. Therefore, it follows that discrepancies may be found not only between the data obtained in different laboratories, but also between data taken in the same laboratory. It should be noted that the author's personal experience on rather simple model systems, namely bacteria and yeasts, is also rich in similar facts. Since mammals are much more complicated than the mentioned organisms, the appearance of discrepancies is not very strange in this sort of experiments. However, the aspiration of scientists to deal with mammals is easy to understand because they are more close to human and often help to resolve some health issues.

During aging increased concentrations of 3-nitrotyrosine, a product of protein nitration, was found in skeletal muscle [79]. The main proteins demonstrating age-dependent accumulation of nitrotyrosine were identified

using Western blotting. They were β-enolase, α-fructose aldolase, creatine kinase, succinate dehydrogenase, triosephosphate isomerase, troponin I, α-crystalline, and glyceraldehyde-3-phosphate dehydrogenase. Since in regulatory proteins evaluated tyrosine residues can be subjected to reversible phosphorylation by tyrosine kinases and phosphatases, their nitration can leave them out of cell control via the mentioned mechanism.

If free radical theory of aging is correct, which is appreciated by many researchers, aging delay might be achieved by either reducing generation or increasing degradation of ROS. The use of antioxidants as one possible way was mentioned above. Caloric restriction without disbalance in micronutrients and vitamins may be the second option. The later helped to demonstrate the possibility to extend not only mean life duration, but in some cases maximal lifespan [80, 81]. For example, the concentrations of protein carbonyls in brain of 15-month-old mice, feed by the standard scheme, could be reduced by caloric restriction during five weeks. On the other hand, the level of protein carbonyls in brain of 15-month-old mice fed by the low-calorie diet was increased after the change from low-calorie to normal rations after five weeks. Therefore, it was concluded that oxidative stress and brain damage in mice is a relatively fast and reversible process [82]. Summarizing, it should be said, that the described pathologies and models of accelerated aging highlighted a clear relationship between aging and accumulation of oxidized proteins [82, 83].

Figure 6 summarizes data on the content of protein carbonyl groups from different tissues of various organisms [84]. It is easy to see that during approximately the last third of life the concentrations of protein carbonyls are significantly increased. That effect does not depend either on organism type or studied tissues, because the given data were obtained with cell culture of human fibroblasts, human eye lens cortex, autopsy samples of human brain tissue, rat liver, and whole houseflies. Over 80% of experimental points were localized in the bordered area. That reflects the global changes in oxidative modification of proteins during aging. Now there are data indicating that during aging mainly specific proteins are accumulated, but here we will not focus on this.

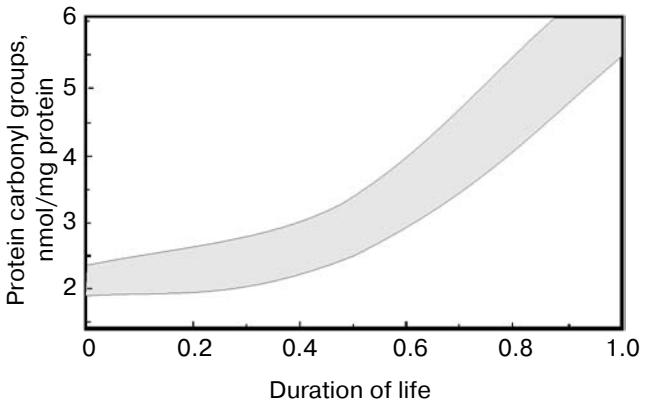

Fig. 6. Concentrations of protein carbonyl groups in tissues of several organisms versus age, represented in semilogarithmic plots. The abscissa is logarithm of life duration, where maximal lifespan is marked as "1". The results were obtained with human skin fibroblasts, human eye lens cortex, autopsy samples of human brain, rat liver, and whole houseflies. Over 80% of the experimental data are localized in the highlighted area (modified from [84]).

Ischemia/reperfusion. Under ischemic conditions, tissue oxygen concentrations are lower than usually. In most cases, it was found that this is accompanied by decreased ROS generation, probably as a consequence of decreased oxygen levels. Simultaneously the intracellular media become more reduced, which is mirrored by higher reduced state of electron transport chain carriers; this may stimulate production of superoxide anion and its metabolites due to one electron reduction by components of the electron transport chain. Ischemia induced by 10-min occlusion of both carotid arteries followed by reperfusion quickly increased concentrations of protein carbonyls in brain of Mongolian gerbil during reperfusion [85]. The strong negative correlation between concentrations of protein carbonyls and activities of glutamine synthase (GS), i.e. higher CP contents, was connected with lower enzyme activities. The spin trap PBN possessing antioxidant properties prevented CP accumulation and decrease in GS activities. The authors suggested that ROS-induced GS-inactivation can result in increase in glutamate concentrations and may be a critical factor resulting in neurotoxicity and brain damage under ischemia/reperfusion [85].

During surgery on the human heart, controlled ischemia followed by reperfusion takes place also. Resulting oxidative stress may cause different disturbances, including arrhythmia. In one experiment, blood samples were taken from the coronary sinus during operation and used to measure concentrations of glutathione, malondialdehyde, and protein carbonyls [61]. All three indices were critically increased during reperfusion, and the calculated correlation coefficients between levels of CP and malondialdehyde were very high ($R^2 > 0.8$). But because CP concentrations were rather stable during reperfusion, at least for 4 h, the authors concluded that this specific parameter can be used to evaluate oxidative stress intensity [61]. Above we already discussed briefly an increase in HNE concentrations in rat hearts subjected to ischemia with subsequent reperfusion. The concentrations of glutathione and the activities of cytochrome *c* oxidase, complex IV, in heart were decreased by 38% simultaneously [30]. Certainly, there are many works on ischemia/reperfusion, but the studies described here help us to understand the main principles of processes taking place. Generally, under limitation or cessation of blood supply, many processes are changed—energy provision is decreased due to inhibition of mitochondria, glycolysis is

activated, and probably ROS generation is decreased. The situation may be partially improved by the increased production of nitric oxide and adenosine, operating as vasodilators, which may redirect blood fluxes to organs critically important for surviving. The ability to survive limitation of blood supply very much depends on the ability to produce energy. It should be noted that under ischemic conditions cell reducing potential, i.e. concentrations of NAD(P)H and GSH and reduction of components of electron transport chain, is enhanced. The later may play a negative role under reoxygenation. The increased ROS generation during reoxygenation, as noted many times by many researchers, i.e. enhanced concentrations of products of ROS-induced oxidation of lipids (malondialdehyde and 4-hydroxynonenal) and proteins (CP) and decreased concentrations of glutathione, creates a critical problem under reoxygenation. The negative consequences of ischemia/reperfusion can be reduced by different antioxidants and previous treatment with weak ischemia/reperfusion (preconditioning). Very similar problems take place during organ transplantation, because transplanted organ is frequently under hypoxic conditions and its connection to the blood system increases oxygen supply accompanied by intensification of ROS production.

Neurodegenerative disorders. Neurodegenerative diseases, namely Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, are a group of age-related disorders. Parkinson's disease is a progressive disorder of the central nervous system. It causes tremors (especially in the hands) and rigidity (especially in the face). Parkinson's disease is characterized by nigral degeneration with Lewy bodies in the pigmented brain stem nuclei, but the contribution of medial *substantia nigra* neuronal loss is unclear, although it is well known that the level of iron ions in *substantia nigra pars compacta* is increased. Memory performance and intellect are not affected. It usually affects people over the age of 50 with subsequent progression. It is supposed that the disease is induced by oxidative stress evidenced by intensification of lipid peroxidation [4, 86]. Earlier we mentioned that during aging CP are accumulated in human tissues. In brain the CP concentration increase depends on the studied part [87]. The age-dependent accumulation of CP is reversibly related to activities of ROS-sensitive enzymes—GS and creatine kinase. Although in Alzheimer disease patients CP concentration was virtually the same as control values, clearly lower GS activities are found [87]. This strongly supports the idea on the use of ROS-sensitive enzymes as markers of oxidative stress.

There is considerable information on the pathway of ROS generation in Parkinson's disease. This processes involves multivalent metal ions (in particular, iron ions), disturbance of electron transport chain functions, end products of glycosylation, reactive microglia, and β-amyloid [88]. The latter is believed to be a chief inducer of the pathology. Amyloid peptides (AP) containing 39-43 amino acid residues are formed from a precursor, a transmembrane glycoproteid. Its neurotoxicity begins after the formation of aggregates with molecular mass over 15 kD. This complex disturbs homeostasis of calcium ions resulting in their accumulation in the cell, and probably is involved in the accumulation of iron ions as well. The accumulation of oxidized proteins in both cytosol and membrane is another sign of induced oxidative stress in Alzheimer's disease [88]. Very serious attention is paid to neurodegenerative diseases due to their broad distribution. It is not surprising that molecular mechanisms of these diseases have been studied in detail at the molecular level. For many different reasons, free radical processes are activated. This results in oxidation of certain proteins, particularly β-amyloid in Parkinson's disease. Partially oxidized proteins can be degraded by proteasome and/or other proteolytic systems. Highly oxidized proteins are not cleaved by proteases and therefore accumulate in cells and can form aggregates. The latter may generate free radicals and also inhibit proteolytic systems, particularly proteasomes [89]. Proteasomes similarly to other proteolytic systems are also subjected to inactivation by ROS. All these events are autocatalytic processes, which progressively damage tissue, stimulating disease development up to death.

Diabetes mellitus. Diabetes mellitus is a very common and widely distributed disease. It is caused either by lack of insulin production by pancreatic β-cells (type I) or lack of insulin receptors at target cells (type II). The development of oxidative stress in β-cells frequently is a prerequisite of decreased insulin production; oxidative stress can damage β-cells or even cause their death. As a result, the concentrations of blood glucose chronically increases, resulting in diabetic complications—blindness, gangrene, damage to kidney and blood vessels, etc. The interaction of glucose with proteins and/or other biomolecules, called the Maillard reaction (formation of brown colored products), is supposed to be a critical point in the development of pathology. An important role is also played by autooxidative glycosylation including glucose autooxidation, formation of bicarbonyl intermediates, and binding of oxidized carbohydrates with proteins [90].

Autooxidation of carbohydrates and products of protein glycosylation, catalyzed by multivalent metal ions, can result in ROS generation, which can further initiate protein damage. The incubation of protein in a system where glucose autooxidation takes place can cause the formation of oxidizing and reducing groups, such as hydroperoxides and hydroxides of aliphatic amino acids, dityrosines, and products of phenylalanine hydroxylation. 2-Glyoxal and arabinose are the main products of glucose autooxidation. The first interacting with proteins gives N^{ε}-(carboxymethyl)lysine, while the second may be involved in generation of fluorescent pentosidin cross-

links in protein. Ascorbate and dicarbonyl carbohydrates such as methyl glyoxal and 3-deoxyglucosone can be involved in autooxidative processes resulting in the formation of brown pigments. Bound to proteins, carbohydrate Amadori intermediates frequently are easier to oxidize than free ones, which may worsen the situation. Both, N^{ε}-(carboxymethyl)lysine and pentosidin are accumulated *in vivo* during aging, and their concentrations are increased in diabetic patients. Most diabetes complication are supposed to be the result of autooxidation of carbohydrates, both free and bound with proteins [90].

Atherosclerosis. Atherosclerosis is characterized by the formation on vessel walls the aggregates of lipids and cells, creating plaques consisting of foam cells. This stimulates cell proliferation, local thickening of the intima, calcium deposition, etc. [1]. The vessel walls became thick and fragile and the arterial lumens are obstructed. The development of atherosclerosis was found to be related with oxidation of macromolecules. About 30% of the cholesterol linoleate deposited in plaques is in oxidized form.

Most deposited lipids originate from low density lipoproteins. The modification of protein apoform by aldehydes and lipid oxidation are key moments in plaque formation in atherosclerosis. Radiolysis and oxidation, catalyzed by metal ions, induce oxidation and fragmentation of the apoprotein. Increased amount of 3-nitrotyrosine relative to apoform was found in plaque proteins *in vivo*. Products of protein oxidation by hypochlorous acid also were found in plaques [60]. Again, it is not clear what is primary and what is a consequence—either formation of atherosclerotic plaques or free radical-induced protein and lipid oxidation. But there is no doubt about the involvement of reactive species in this pathology.

Several other pathologies are related with free radical-induced protein oxidation. For example, the oxidation of the plasma protein fibrinogen results in its inability to the structural form of blood clots, its ability to form clots negatively correlating with the oxidation level [91]. Oxidation of immunoglobulin by synovial liquor induces their aggregation, which is supposed to be associated with arthritis [92]. The oxidation of protease inhibitors such as α-1-antitrypsin results is several physiological consequences. For example, the oxidation of a critical methionine residue in α-1-antitrypsin leads to its inactivation, and since this inhibitor protein is responsible for inhibition of proteases in tissues, mainly in lungs and cartilage, this process is believed to be related to tissue damage in emphysema [93]. Oxidation can be caused by hypochlorous acid produced by activated neutrophils [94]. Cataractogenesis can be also associated with oxidation of the crystalline lens of the eye [95-97]. Increased amount of oxidized proteins are found in several other pathologies, but in most cases specific proteins have not been identified.

Oxidative stress—bacteria and lower vertebrates. From the material described above the false impression might be given that oxidative damage of proteins is a sign or studied only in humans and other mammals. That is not the case. For example, the first detailed mechanism of response to oxidative stress—an increase of steady-state ROS concentrations—was studied in enterobacteria [1, 3]. It was shown that oxidative stress increased the efficiency of protective systems, particularly the activities of antioxidant and associated enzymes. We have analyzed this problem earlier [53]. Here we will focus only on selected works where carbonyl groups of proteins were used as markers of oxidative stress.

In our laboratory, concentrations of protein carbonyls along with other markers of oxidative stress have been used for several years. The addition of hydrogen peroxide to media with the enterobacterium *Escherichia coli* induced accumulation of protein carbonyls [98]. The protein carbonyl concentrations gradually increased for 60 min, while concentrations of other oxidative stress markers, so-called thiobarbituric reactive substances (TBARS) first increased, but further decreased. Therefore, it was concluded that on investigation, several markers of oxidative stress should be used, and the level of protein carbonyls cannot be the only parameter of choice. The additional markers, which can be used to evaluate oxidative stress intensity, are, for example, concentrations of lipid peroxides, the ratio of oxidized and reduced glutathione, the activities of enzymes directly or not directly involved in the protection or detoxication of ROS or products of their interaction with cell components, reparation of damage, etc. The intensity of response to oxidative stress very much depended on the bacterial strain used. For example, some indices such as enzyme activities in cells of certain strains could not be changed under stress conditions [98]. Therefore, one can conclude that the concentration of oxidized proteins is a reliable marker of oxidative stress in bacteria. This sort of response can help bacteria to survive under unfavorable conditions, which are frequently accompanied by oxidative stress. These conditions include temperature changes, treatment by antibiotics, or attack by cells of the immune system.

Lower vertebrates are also subjected to oxidative stress. It can be a component of any stress, such as induced by temperature change and oxygen availability, physical exercise, toxicant effects, etc. Therefore, for about 10 years we study the effects of external factors on free radical processes in fish and frogs. The concentration of protein carbonyls was found to be a good marker of oxidative stress in animals, but here also the above-described features are correct. Frogs *Rana ridibunda*, collected in autumn at low environmental temperatures, were kept under hibernation conditions at 2-4°C. Further, the environmental temperature was increased to 20°C and several markers of oxidative stress, such as protein carbonyls, TBARS, as well the activities of antioxidant and

associated enzymes were evaluated [62]. It should be noted that the metabolism intensity in exothermal animals in the physiological range increases proportionally to growth temperature. Since it is well known that metabolism intensification stimulates the production of ROS as side products, one could expect the development of oxidative stress on the transition from hibernation to active state. In fact, we found not only the increase in protein carbonyl concentrations, but also enhancement of TBARS concentrations and activities of antioxidant and associated enzymes, namely superoxide dismutase, catalase, glutathione reductase, and glucose-6-phosphate dehydrogenase [62]. With another animal frequently used for studies, the cold-blooded goldfish *Carassius auratus*, we investigated the effect of temperature increase from 20 to 35°C and found that the protein carbonyl concentrations also changed, but its presence or manifestation greatly depended on the studied tissue type [99].

The effect of increased environmental oxygen concentration (hyperoxia) on oxidative stress markers in several goldfish tissues was studied also [100]. Again, the responses were very tissue-specific, but in all investigated tissues, namely brain, liver, kidney, and muscle, the parameters were increased, but to different extent. Interestingly, certain dynamic relationships between concentrations of protein carbonyls and concentrations of lipid peroxides or TBARS were found. During experiments, protein carbonyls gradually accumulated [100]. The results can be interpreted in the light of earlier described pathways of additional protein carbonyl formation under oxidative stress. Lipid peroxides and products of their metabolism, including aldehydes evaluated as TBARS, may be responsible for the formation of additional protein carbonyl groups.

At first glance, hypoxia may be thought to be opposite to hyperoxia. However, the picture is more complicated. Using the common carp *Cyprinus carpio* we investigated the effects of hypoxia and recovery on parameters of oxidative stress [101]. Again, effects were tissue-specific. Since in this review we focus on protein oxidation, it should be said that under hypoxic conditions the concentrations of CP in liver and brain were unchanged and in kidney even slightly decreased. The levels of lipid peroxides were reduced in brain and liver, but not changed in kidney and muscle, and TBARS concentrations in liver enhanced. Reoxygenation for 14 h increased protein carbonyl concentrations in liver, and in kidney and muscle tendency to restore the initial level was found. It should be noted that oxidized lipids cannot only be metabolized by specific enzymes, but also interact with proteins forming additional carbonyl groups. The activities of antioxidant enzymes were either decreased or unchanged during hypoxia [101]. Therefore, it can be concluded that in our hands under hypoxic conditions the intensity of ROS generation reduced, which is in good agreement with many conducted studies. Moreover, it cannot be excluded that decreased markers of oxidative stress under hypoxia may lead to development of reductive stress, the state opposite to oxidative stress. However, this point of view needs further development.

SOME EXAMPLES OF *in vivo* PROTECTION OF PROTEINS AGAINST OXIDATION

In this chapter, we will briefly focus on some examples of protection of proteins against oxidation in whole cells. These examples are best demonstrated using microorganism models. On studying the role of mitochondrial NAD^+ kinase of baker's yeast *Saccharomyces cerevisiae*, it was shown that inactivation of the *POS5* gene, coding for the mentioned enzyme, resulted in 28-fold increase in protein carbonyl concentrations in mitochondria [102] and many times lower activities of oxidation-sensitive enzymes, namely aconitase and succinate dehydrogenase, compared to the parental strain [103]. We also studied some markers of oxidative stress in *S. cerevisiae* using different strains—parental and isogenic derivatives defective in one of two or in both catalases of the yeast [104]. It was found that higher catalase activities corresponded to lower protein carbonyl levels (Fig. 7). Very strong positive correlation between the activities of catalase and activities of the oxidation-sensitive enzymes glucose-6-phosphate dehydrogenase and glutathione reductase was found. It follows that higher catalase activities correspond to lower protein carbonyl levels, while the activities of oxidation-sensitive enzymes demonstrate the opposite relationship. Rather strong negative correlation between catalase activities and protein carbonyl concentrations, very similar to one described above for yeast, was found in brain of goldfish treated by the catalase inhibitor pesticide aminotriazole [105]. Although results of correlative analysis cannot be used as strong proof of a protective role of catalase toward cellular proteins, many experiments that have been performed give some clues to suggest that *in vivo* catalase may protect cellular proteins from ROS-induced oxidation. This suggestion can be supported also by our results with yeast glucose-6-phosphate dehydrogenase [106]. *In vitro* hydrogen peroxide inactivated the partially purified enzyme, which confirmed our data obtained *in vivo* on possible catalase involvement in the enzyme regulation in the whole cell. Under these conditions, the enzyme was modified in several stages: first it was partially inactivated with a change in kinetic characteristics, and later it lost activity completely.

Using yeast, we also investigated a protective role of superoxide dismutases. Several approaches were used, and in all cases the relationship between the activities of superoxide dismutase and protein carbonyl groups was evaluated. In one experiment several yeast strains were used, a parental type expressing both superoxide dismu-

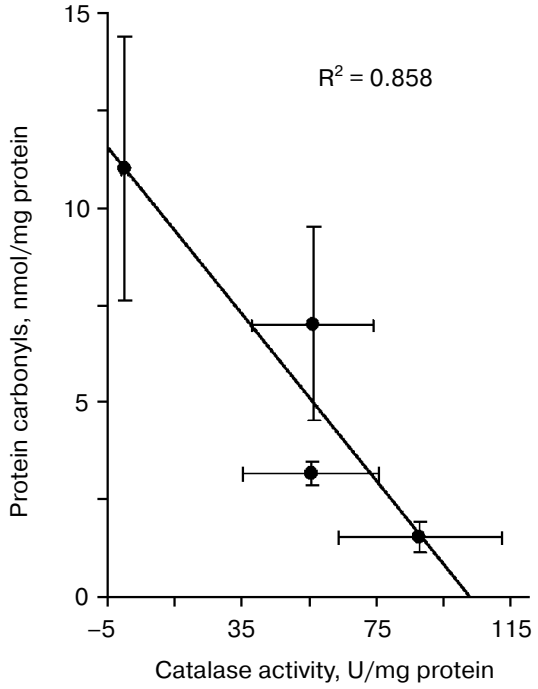

Fig. 7. Relationship between catalase activities and contents of carbonyl groups in protein fraction of cell-free extracts of yeast *Saccharomyces cerevisiae* in parental strain and strains defective in either one or two catalases possessed by this organism (modified from [101]).

tases yeast possess, and isogenic derivatives defective either in Cu,Zn- or Mn-containing or in both superoxide dismutases [107]. The observed dependence was very close to parabolic. It seems that depending on the activity superoxide dismutase can be either a pro- or anti-oxidant. Earlier J. McCord and colleagues found that *in vitro* superoxide dismutase can be a pro-oxidant [108]. In the cited work [107], we also noted another interesting fact—a strong positive correlation between the activities of catalase and superoxide dismutase, which may reflect a coordination of antioxidant systems under the experimental conditions used. In further work we used the yeast strain defective in Mn-containing superoxide dismutase and inhibited Cu,Zn-containing superoxide dismutase with diethyldithiocarbamate [109]. The conditions used were slightly different from those used in previous work, and results on the first view were totally opposite. The relationship between protein carbonyl concentrations and superoxide dismutase activities was bell-shaped (Fig. 8). A strong positive correlation between the activities of catalase and superoxide dismutase was found again [109]. A rather similar relationship between the activities of catalase and superoxide dismutase was found on investigation of hydrogen peroxide effects on yeast [110]. Therefore, we concluded that *in vivo* in several different experimental models both catalases and superoxide dismutases can protect proteins from ROS-induced oxidation, although this relationship is not always linear. We analyzed the free radical-induced protein oxidation in baker's yeast in a special review [111].

Figure 9 summarizes a modern view on the involvement of ROS in oxidation of cellular proteins. The intensity of oxidation can depend on interaction with certain ligands, for example substrates or cofactors, which may partially prevent oxidative modification. Different

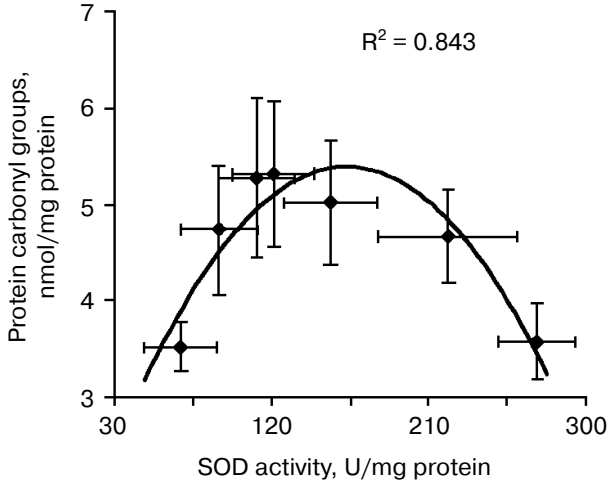

Fig. 8. Relationship between content of protein carbonyls and superoxide dismutase activity in cell-free extract of *Saccharomyces cerevisiae* yeast strain defective in Mn-containing superoxide dismutase on inhibition of Cu,Zn-containing superoxide dismutase by diethyl dithiocarbamate (modified from [109]).

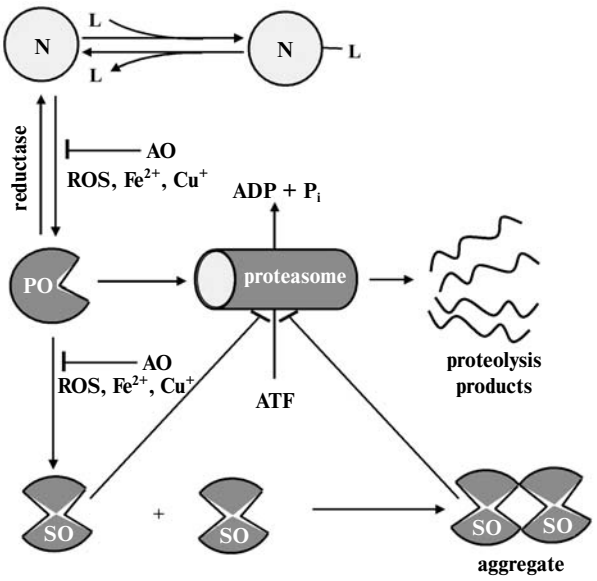

Fig. 9. Formation and intracellular processing of oxidized proteins. Explanations are in the text. Designations: N, normal protein; L, ligand; PO and SO, partially and strongly oxidized proteins; AO, antioxidants.

antioxidants can protect proteins also. At least three scenarios of events can occur after protein oxidation: first, it can be repaired to the initial form in the case when cysteine residue was oxidized (formation of sulfenic acid residue, cystine or mixed disulphide) or methionine (formation of methionine sulfoxide); the second—degradation via ATP-dependent pathway either by proteasomes or specific proteases, which result in the formation of respective peptides; and the third—further oxidation of partially oxidized protein, which can lead to aggregate formation. The latter cannot only be accumulated in the cell, but substantially affect cellular processes, for example, to inhibit proteasomes [89]. The listed processes can form a feedback link that worsens the state of a cell subjected to oxidative stress. Several new works have demonstrated that free radical-induced oxidation of certain cellular proteins can induce apoptosis [112-116].

T. Nystrom and colleagues have carried out an interesting series of works on the relationship between free radical protein oxidation and the physiological state of individual cells or whole organisms. Using budding yeast *S. cerevisiae*, they found that not depending on "age" of the maternal cell and on the amount of protein carbonyl groups, the level of oxidized proteins in new budded cells it always rather low [117]. Using the classical plant model *Arabidopsis thaliana*, it was found that during plant development till the flowering phase protein carbonyl levels increased about 5-fold, but on entering the flowering phase (16-18th day) it sharply decreased to that of 8-day-old plants and was further maintained at least until day 36 [118]. The chlorophyll content was virtually unchanged starting from day 15 till the end of the experiment. On the study of embryonic ES stem cells, this team unexpectedly found that they contain high levels of protein carbonyls and products of protein glycation [119]. Chaperones and cytoskeletal proteins were the main oxidized proteins. Cell differentiation decreased protein carbonyl concentrations. The authors suggested that a previously unknown process of revitalization at the protein level takes place in the early phases of embryonal development. In an excellent review on the role of free radical protein oxidation, T. Nystrom summarized different scenarios of dynamics of oxidized proteins in various organisms. He concluded that there are several strategies in early development, maturation, reproduction, and aging, but the information for a general view is still scarce [120].

CONCLUSIONS AND PERSPECTIVES

At present the level of our knowledge about processes of free radical oxidation of proteins becomes clear—that they may have not only negative consequences, but are also involved in regulation of many cellular processes. The discovery of pathways for specific degradation of oxidized proteins, particularly ATP-dependent hydrolysis by specific proteases or proteasome, radically changed our view of these processes. Although protein oxidation takes place as an "occasional" process, it is involved in regulation of metabolism. Something similar was found earlier for lipids. The possibility to reduce oxidized residues of sulfur-containing amino acids, cysteine and methionine, is used in regulation of many cellular processes. There is a large system of specific regulatory proteins that sense ROS, and their reversible oxidation is involved in regulation of gene expression. Oxidation of proteins also can take part in regulation of spatial-temporal protein distribution in cells.

Although much is known about chemical mechanisms of protein oxidation, this cannot be said about functional consequences, although many works have been carried out in these directions also. So, *in vitro* it was found that oxidation can affect catalytic and regulatory properties up to full inactivation. At the level of whole cells, to say something certain is rather complicated. Some progress in the field of *in vivo* protein oxidation and protection can be reached using correlative analysis of relationships between the activities of specific enzymes, concentrations of products of free radical-induced modification of proteins, and the activities of antioxidant enzymes. However, further investigations concerning these factors are also needed.

There are no doubts about a relationship between the levels of oxidized proteins and certain functional states of organisms. So, aging, hyper- and hypoxia, temperature increase, some pathologies such as cardiovascular diseases, ischemia/reperfusion, diabetes, and neurodegenerative pathologies, are clearly connected with increased levels of oxidized proteins. Although it is still impossible to state that the listed changes are consequences of protein oxidation, in some cases there are clear evidences on that. Many examples demonstrate that the decrease in oxidized proteins correlated with improvement. Therefore, it is widely believed that the level of oxidized proteins at least may be a good marker of mentioned changes and can be used for the development of preventive approaches. The study of dynamics of an organism's state and the levels of oxidized proteins, lipids, and nucleic acids may help to respond to the question of causes and relationships between the listed processes. For example, in our works this approach revealed that under oxidative stress, first the level of oxidized lipids is increased, which is followed by protein oxidation. This seems logical, since, as noted earlier, certain products of lipid oxidation can interact with proteins forming additional carbonyl groups. The study of the character and dynamics of products of free radical protein oxidation may have prognostic value in certain human and animal pathologies and be used for prophylactic and treatment.

The author is grateful to Drs. H. Semchyshyn and T. Bagnyukova for critical reading of the manuscript, O.

Lushchak for assistance in the preparation of figures, and Drs. D. A. Butterfield, A. A. Boldyrev, V. I. Bunik, R. Weindruch, K. J. Davies, R. L. Levine, J. M. McCord, K. Pantopoulos, L. I. Szweda, V. P. Skulachev, R. S. Sohal, E. R. Stadtman, D. Touati, I. Fridovich, and S. M. Jazwinski for the sending reprints. Special thanks to the anonymous referee for the useful comments and recommendations which helped to improve the manuscript.

REFERENCES

1. Halliwell, B., and Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford.
2. Skulachev, V. P. (1996) *Quart. Rev. Biophys.*, **29**, 169-202.
3. Johnson, P., and Boldyrev, A. A. (2002) *Oxidative Stress at Molecular, Cellular and Organ Levels*, Research Signpost, India.
4. Boldyrev, A. A. (1995) *Biochemistry (Moscow)*, **60**, 1173-1178.
5. Kuo, C. F., Mashino, T., and Fridovich, I. (1987) *J. Biol. Chem.*, **262**, 4724-4727.
6. Gardner, P. R., and Fridovich, I. (1991) *J. Biol. Chem.*, **266**, 19328-19333.
7. Brown, N. M., Kennedy, M. C., Antholine, W. E., Eisenstein, R. S., and Walden, W. E. (2002) *J. Biol. Chem.*, **277**, 7246-7254.
8. Stadtman, E. R. (1993) *Annu. Rev. Biochem.*, **62**, 797-821.
9. Dean, R. T., Fu, S., Stocker, R., and Davies, M. J. (1997) *Biochem. J.*, **324**, 1-18.
10. Stadtman, E. R. (1998) in *Free Radicals, Oxidative Stress, and Antioxidants* (Ozben, ed.) Academic Plenum Press, N. Y., pp. 51-64.
11. Stadtman, E. R., and Levine, R. L. (2003) *Amino Acids*, **25**, 207-218.
12. Schuessler, H., and Giesseg, S. P. (1984) *Int. J. Radiat. Biol.*, **45**, 267-281.
13. Levine, R. L., Wehr, N., Williams, J. A., Stadtman, E. R., and Shacter, E. (2000) *Meth. Mol. Biol.*, **99**, 15-24.
14. Lushchak, V. I., Bagnykova, T. V., and Lushchak, O. V. (2004) *Ukr. Biokhim. Zh.*, **76**, 136-141.
15. Levine, R. L., Moskovitz, J., and Stadtman, E. R. (2000) *Life*, **50**, 301-307.
16. Thomas, J. A., and Mallis, R. J. (2001) *Exp. Gerontol.*, **36**, 519-526.
17. Stadtman, E. R., Moskovitz, J., and Levine, R. L. (2003) *Antioxidants and Redox Signaling*, **5**, 577-582.
18. Moskovitz, J., Berlett, B. S., Poston, J. M., and Stadtman, E. R. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 9585-9589.
19. Cervera, J., and Levine, R. L. (1988) *FASEB J.*, **2**, 2591-2595.
20. Levine, R. L., Mosoni, L., Berlett, B. S., and Stadtman, E. R. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 15036-15040.
21. Lushchak, V. I. (2002) *Vopr. Biol. Med. Farmakol. Khim.*, **1**, 27-31.
22. Lushchak, V. I. (1996) *Biochemistry (Moscow)*, **61**, 143-154.
23. Uchida, K., and Stadtman, E. R. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 5611-5615.
24. Uchida, K., and Stadtman, E. R. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 4544-4548.
25. Uchida, K., Szweda, L. I., Chae, H.-Z., and Stadtman, E. R. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 8742-8746.
26. Uchida, K., and Stadtman, E. R. (1993) *J. Biol. Chem.*, **268**, 6388-6393.
27. Uchida, K., and Stadtman, E. R. (1994) *Meth. Mol. Biol. Stress Response: Methods and Protocols* (Keyse, ed.) Vol. 99, pp. 25-34.
28. Uchida, K., Itakura, K., Kawakishi, S., Hiai, H., Toyokuni, S., and Stadtman, E. R. (1995) *Arch. Biochem. Biophys.*, **324**, 241-248.
29. Szweda, L. I., Uchida, K., Tsai, L., and Stadtman, E. R. (1993) *J. Biol. Chem.*, **268**, 3342-3347.
30. Chen, J., Henderson, G. I., and Freeman, G. L. (2001) *J. Mol. Cell. Cardiol.*, **33**, 1919-1927.
31. Stadtman, E. R. (1991) *XXXV Chemistry at the Frontiers of Medicine*, Houston, TX, pp. 182-203.
32. Levine, R. L. (1983) *J. Biol. Chem.*, **258**, 11823-11827.
33. Levine, R. L., Oliver, C. N., Fulks, R. M., and Stadtman, E. R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2120-2124.
34. Levine, R. L. (1983) *J. Biol. Chem.*, **258**, 11828-11833.
35. Stadtman, E. R., and Wittenberger, M. E. (1985) *Arch. Biochem. Biophys.*, **239**, 379-387.
36. Kim, K., Rhee, S. G., and Stadtman, E. R. (1985) *J. Biol. Chem.*, **260**, 15394-15397.
37. Farber, J. M., and Levine, R. L. (1986) *J. Biol. Chem.*, **261**, 4574-4578.
38. Roseman, J. E., and Levine, R. L. (1987) *J. Biol. Chem.*, **262**, 2101-2110.
39. Levine, R. L., and Rivett, A. J. (1989) *Medical, Biochemical, and Chemical Aspects of Free Radicals* (Hayaishi, O., Kondo, M., and Yoshikawa, T., eds.) Elsevier Science Publishers, pp. 1195-1202.
40. Rivett, A. J., and Levine, R. L. (1990) *Arch. Biochem. Biophys.*, **278**, 26-34.
41. Climent, I., and Levine, R. L. (1991) *Arch. Biochem. Biophys.*, **289**, 371-375.
42. Fisher, M. T., and Stadtman, E. R. (1992) *J. Biol. Chem.*, **267**, 1872-1880.
43. Berlett, B. S., Friguet, B., Yim, M. B., Chock, P. B., and Stadtman, E. R. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 1776-1780.
44. Berlett, B. S., Levine, R. L., and Stadtman, E. R. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 2784-2789.
45. Ma, Y.-S., Chao, C.-C., and Stadtman, E. R. (1999) *Arch. Biochem. Biophys.*, **363**, 129-134.
46. Stadtman, E. R., Oliver, C. N., and Starke-Reed, P. E. (1991) *Korean J. Biochem.*, **23**, 49-54.
47. Szweda, L. I., and Stadtman, E. R. (1992) *J. Biol. Chem.*, **267**, 3096-3100.
48. Szweda, L. I., and Stadtman, E. R. (1993) *Arch. Biochem. Biophys.*, **301**, 391-395.
49. Nelson, S. R., Pazdernik, T. L., and Samson, F. E. (1992) *Proc. West. Pharmacol. Soc.*, **35**, 37-41.
50. Liochev, S. I., Benov, L., Touati, D., and Fridovich, I. (1999) *J. Biol. Chem.*, **274**, 9479-9481.
51. Imlay, J. A. (2003) *Annu. Rev. Microbiol.*, **57**, 395-418.
52. Touati, D., Jacques, M., Tardat, B., Bouchard, L., and Despied, S. (1995) *J. Bacteriol.*, **177**, 2305-2314.
53. Lushchak, V. I. (2001) *Biochemistry (Moscow)*, **66**, 476-489.
54. Nulton-Person, A. C., and Szweda, L. I. (2001) *J. Biol. Chem.*, **276**, 23357-23361.
55. Haile, D. J., Rouault, T. A., Harford, J. B., Kennedy, M. C., Blondin, G. A., Beinert, H., and Klausner, R. D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 11735-11739.

56. Fillebeen, C., and Pantopoulos, K. (2002) *Redox Report.*, **7**, 15-22.
57. Lucas, D. T., and Szweda, L. I. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 6689-6693.
58. Bunik, V. I., and Sievers, C. (2002) *Eur. J. Biochem.*, **269**, 5004-5015.
59. Bunik, V. I. (2003) *Eur. J. Biochem.*, **270**, 1036-1042.
60. Shacter, E. Y. (2000) *Drug Metab. Rev.*, **32**, 307-326.
61. Pantke, U., Volk, T., Schmutzler, M., Kox, W. J., Sitte, N., and Grune, T. (1999) *Free Rad. Biol. Med.*, **27**, 1080-1086.
62. Bagnyukova, T. V., Storey, K. B., and Lushchak, V. I. (2003) *J. Therm. Biol.*, **28**, 21-28.
63. Stadtman, E. R. (1995) *Molecular Aspects of Aging* (Esser, K., and Martin, G. M., eds.) John Wiley & Sons, Ltd., pp. 129-143.
64. Stadtman, E. R., and Berlett, B. S. (1999) in *Reactive Oxygen Species in Biological Systems* (Gilbert and Colton, eds.) Vol. 27, Kluwer Academic/Plenum Publishers, N. Y., pp. 657-675.
65. Stadtman, E. R., Oliver, C. N., Starke-Reed, P. E., and Rhee, S. G. (1993) *Toxicol. Ind. Health.*, **9**, 187-196.
66. Stadtman, E. R. (1988) *J. Gerontol.: Biol. Sci.*, **43**, B112-120.
67. Sohal, R. S. (2002) *Free Rad. Biol. Med.*, **33**, 37-44.
68. Stadtman, E. R., and Berlett, B. S. (1998) *Drug Metab. Rev.*, **30**, 225-243.
69. Sohal, R. S., Agarwal, S., Dubey, A., and Orr, W. C. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 7255-7259.
70. Farmer, K. J., and Sohal, R. S. (1989) *Free Rad. Biol. Med.*, **7**, 23-29.
71. Sohal, R. S., and Dubey, A. (1994) *Free Rad. Biol. Med.*, **16**, 621-626.
72. Dubey, A., Forster, M. J., and Sohal, R. S. (1995) *Arch. Biochem. Biophys.*, **324**, 249-254.
73. Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Landum, R. W., Cheng, M. S., Wu, J. F., and Floyd, R. A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 3633-3636.
74. Forster, M. J., Dubey, A., Dawson, K. M., Stutts, W. A., Lal, H., and Sohal, R. S. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4765-4769.
75. Oliver, C. N., Ahn, B.-W., Moerman, E. J., Goldstein, S., and Stadtman, E. R. (1987) *J. Biol. Chem.*, **262**, 5488-5491.
76. Cabiscol, E., and Levine, R. L. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 4170-4174.
77. Sahakian, J. A., Szweda, L. I., Friguet, B., Kitani, K., and Levine, R. L. (1995) *Arch. Biochem. Biophys.*, **318**, 411-417.
78. Starke-Reed, P. E., and Oliver, C. N. (1989) *Arch. Biochem. Biophys.*, **275**, 559-567.
79. Kanski, J., Alterman, M. A., and Schoneich, C. (2003) *Free Rad. Biol. Med.*, **35**, 1229-1239.
80. Sohal, R. S., and Weindruch, R. (1996) *Science*, **273**, 59-63.
81. Jazwinski, S. M. (1996) *Science*, **273**, 54-74.
82. Butterfield, D. A., Howard, B. J., Yatin, S., Allen, K. L., and Carney, J. M. (1997) *Proc. Natl. Acad. Sci. USA*, **94**, 674-678.
83. Yuneva, M. (2002) *Mechanisms of Oxidative Stress in Senescence Accelerated Mice in Oxidative Stress at Molecular, Cellular and Organ Levels* (Johnston, P., and Boldyrev, A., eds.) Research Singpost, Trivandrum, India, pp. 131-142.
84. Levine, R. L., and Stadtman, E. R. (2000) in *Science of Geriatrics* (Morley, J. E., Armbrecht, H. J., Coe, R. M., and Vellas, B., eds.) Serdi Publisher, Paris, Springer Publishing Company, N. Y., pp. 75-94.
85. Oliver, C. N., Starke-Reed, P. E., Stadtman, E. R., Liu, G. J., Carney, J. M., and Floyd, R. A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5144-5147.
86. McCord, J. M. (1998) *Seminars Hematol.*, **35**, 5-12.
87. Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A., and Markesbery, W. R. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10540-10543.
88. Butterfield, D. A., Hensley, K., Hall, N., Subramaniam, R., Howard, B. J., Cole, P., Yatin, S., LaFontaine, M., Harris, M. E., Aksenova, M., Aksenov, M., and Carney, J. M. (1996) in *Molecular Mechanisms of Dementia* (Wasco, W., and Tanzi, R. E., eds.) Humana Press Inc., Totowa, NJ, pp. 145-167.
89. Grune, T. (2000) *Biogerontology*, **1**, 31-40.
90. Hunt, J. V., Bottoms, M. A., and Mitchinson, M. J. (1993) *Biochem. J.*, **291**, 529-535.
91. Shacter, E., Williams, J. A., and Levine, R. L. (1995) *Free Rad. Biol. Med.*, **18**, 815-821.
92. Jasin, H. E. (1983) *J. Immunol.*, **130**, 1918-1923.
93. Carp, H., Miller, F., Hoidal, J. R., and Janoff, A. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2041-2045.
94. Matheson, N. R., Wong, P. S., and Travis, J. (1979) *Biochem. Biophys. Res. Commun.*, **88**, 402-409.
95. Garland, D. (1990) *Exp. Eye Res.*, **50**, 677-682.
96. Davies, K. J. A. (1990) *Adv. Exp. Med. Biol.*, **264**, 503-511.
97. Murakami, K., Jahngen, J. H., Lin, S. W., Davies, K. J., and Taylor, A. (1990) *Free Rad. Biol. Med.*, **8**, 217-222.
98. Semchyshyn, H. M. (2002) *Biochemical Peculiarities of Escherichia coli Strains with Different Sensitivity to Oxygen*: Thesis of Ph. D. dissertation, Chernivtsi National University, Chernivtsi.
99. Lushchak, V. I., and Bagnyukova, T. V. (2006) *Comp. Biochem. Physiol.*, **144**, 283-289.
100. Lushchak, V. I., Bagnyukova, T. V., Husak, V. V., Luzhna, L. I., Lushchak, O. V., and Storey, K. B. (2005) *Int. J. Biochem. Cell Biol.*, **37**, 1670-1680.
101. Lushchak, V. I., Bagnyukova, T. V., Lushchak, O. V., Storey, J. M., and Storey, K. B. (2005) *Int. J. Biochem. Cell. Biol.*, **37**, 1319-1330.
102. Strand, M. K., Stuart, G. R., Longley, M. J., Graziewicz, M. A., Dominick, O. C., and Copeland, W. C. (2003) *Eukaryotic Cell*, **2**, 809-820.
103. Jensen, L. T., Sanchez, R. J., Srinivasan, C., Valentine, J. S., and Culotta, V. C. (2004) *J. Biol. Chem.*, **279**, 29938-29943.
104. Lushchak, V. I., and Gospodaryov, D. V. (2005) *Cell. Biol. Int.*, **29**, 187-192.
105. Bagnyukova, T. V., Vasylkiv, O. Yu., Storey, K. B., and Lushchak, V. I. (2005) *Brain Res.*, **1052**, 180-186.
106. Gospodarev, D. V., Bailyak, M. M., and Lushchak, V. I. (2005) *Ukr. Biokhim. Zh.*, **77**, 58-64.
107. Lushchak, V., Semchyshyn, H., Mandryk, S., and Lushchak, O. (2005) *Arch. Biochem. Biophys.*, **441**, 35-40.
108. Nelson, K., Bose, S. K., and McCord, J. M. (1994) *Free Rad. Biol. Med.*, **16**, 195-200.
109. Lushchak, V., Semchyshyn, H., Lushchak, O., and Mandryk, S. (2005) *Biochem. Biophys. Res. Commun.*, **338**, 1739-1744.

110. Bailyak, M. V., Semchyshyn, H. M., and Lushchak, V. I. (2006) *Biochemistry (Moscow)*, **71**, 1013-1020.
111. Lushchak, V. I. (2006) *Acta Biochim. Polonica*, **53**, 762-771.
112. Dalle-Donne, I., Aldini, G., Carini, M., Colombo, R., Rossi, R., and Milzani, A. (2006) *J. Cell. Mol. Med.*, **10**, 389-406.
113. England, K., O'Driscoll, C., and Cotter, T. G. (2004) *Cell. Death Differ.*, **11**, 252-260.
114. Powell, S. R., Wang, P., Divald, A., Teichberg, S., Haridas, V., McCloskey, T. W., Davies, K. J., and Katzeff, H. (2005) *Free Rad. Biol. Med.*, **38**, 1093-1101.
115. Ding, Q., and Keller, J. N. (2001) *Free Rad. Biol. Med.*, **31**, 574-584.
116. Cahuana, G. M., Tejedo, J. R., Jimenez, J., Ramirez, R., Sobrino, F., and Bedoya, F. J. (2003) *Biochem. Pharmacol.*, **66**, 1963-1971.
117. Aguilaniu, H., Gustafsson, L., Rigoulet, M., and Nystrom, T. (2003) *Science*, **299**, 1751-1753.
118. Johansson, E., Olsson, O., and Nystrom, T. (2004) *J. Biol. Chem.*, **279**, 22204-22208.
119. Hernebring, M., Brolen, G., Aguilaniu, H., Semb, H., and Nystrom, T. (2006) *Proc. Nat. Acad. Sci. USA*, **103**, 7700-7705.
120. Nystrom, T. (2005) *EMBO J.*, **24**, 1311-1317.