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Isotope Effect, Essential Diet Components, and Prospects of Aging Retardation

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Abstract—A method is proposed that has the potential to increase the stability of lipids, proteins, nucleic acids and other cellular components towards the detrimental damages caused by reactive oxygen species (ROS). The rate-limiting step of most ROS-driven oxidation reactions is hydrogen abstraction. The oxidation-susceptible sites within these (bio)molecules can thus be made less vulnerable to ROS-driven oxidation by incorporating heavy stable isotopes, such as deuterium or/and carbon-13. Ingestion of isotopically reinforced building blocks, such as amino acids, lipids, and components of nucleic acids and their subsequent incorporation into macromolecules would make the latter more stable to ROS courtesy of the isotope effect. The suggested approach may lead to enhanced resistance toward oxidative stress and, hence, to enhanced longevity.

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"Reinforcing" biomolecules due to an isotope effect with the aim of protecting body cells and tissues from harmful factors can play an important role in the aging retardation process. Before addressing this subject, let us dwell shortly on isotope effects.

A classical isotope effect is the effect produced by substitution of a light isotope in a molecule with a heavy isotope (for example, substitution of protium by deuterium) on chemical bond energy and, as a result, on reaction rate (kinetic isotope effect). The vibrational energy of a bond in the ground state of a molecule is the lower the higher the reduced mass of the atoms forming this bond. Thus, the chemical bond between heavy isotopes has a lower energy in the ground molecular state, and the dissociation energy of this bond is higher.

The primary and secondary isotope effects have different impacts on reaction rate [1]. The primary kinetic isotope effect arises when bond dissociation or formation occurs in the transition state of the reaction (rate-limiting stage). When the heavy isotope locates in the vicinity of the dissociating bond, the secondary isotope effect takes place, and it is much weaker than primary. The equilibrium isotope effect is observed in molecular exchange, for example, in the exchange between the atmospheric CO_2 and marine CO_2 (hydrocarbonate): In the equilibrium at 25°C, air is empoverished in ^{13}C by 11‰ [2]. The magnetic isotope effect depends on nuclear spin, and, manifesting itself in reactions involving radical pairs, leads to fractionation of magnetic and nonmagnetic nuclei [3]. The isotope effect is pressure- and temperature-dependent.

Of the greatest interest in the context of this paper are stable isotopes which are abundant in the nature and their induced kinetic isotope effect is fairly strong. There requirements are primarily met by deuterium and, in part, by ^{13}C, ^{15}N, and ^{18}O (see table).

The free-radical theory of aging, suggested in 1956 [7] and presently widely, even though not completely, recognized [8] explains the irreversible changes in cells and decline in the efficiency of cellular processes by the hazardous effect of ROS which damage DNA, proteins, lipids, and other cellular components. A strong correlation was revealed between aging, age-related diseases, oxidative stress, and oxidative damages [9].

The ROS include oxygen radicals (superoxide $O_2^{-\bullet}$, hydroperoxyl $HO_2^{\bullet}$, hyroxyl $HO^{\bullet}$, peroxyl $ROO^{\bullet}$,

Isotope effects (IE) of different isotopes[a]

Isotope	Natural abundance, mol %	Theoretical [4] and actual IE	Nuclear spin I [5]	Natural scatter of isotope ratios
^{1}H(H), stable	0.99985	1	1/2	
^{2}H(D), stable	0.00015	18 (CH/CD, 6–8)	1	D/H = 250%
^{3}H(T), $\tau/2$ = 12.26 years	$<10^{-17}$	60 (CH/CT, 15–16)	1/2	
^{11}C, $\tau/2$ = 20.3 min	–	–	3/2	
^{12}C, stable	0.9889	1	0	
^{13}C, stable	0.0111	1.25 (^{12}C/^{13}C, 1.06)	1/2	^{13}C/^{12}C = 100%
^{14}C, $\tau/2$=5730 years	$<10^{-12}$	1.5 (^{12}C/^{14}C, 1.08)	0	
^{16}O, stable	0.99759	1	0	
^{17}O, stable	0.00037	—	5/2	^{18}O/^{16}O = 100%
^{18}O, stable	0.00204	1.19 (^{16}O/^{18}O, 1.03)	0	
^{14}N, stable	0.99632	1	1	
^{15}N, stable	0.00368	1.14 (^{14}N/^{15}N, 1.04)	1/2	^{15}N/^{14}N = 100%

[a] Given are data for the most abundant cellular elements: H (cellular fraction 0.1), C (0.18), O (0.65), N (0.03); data for phosphorus (0.012) and sufur (0.002) can be found in [6].

alkoxyl $RO^{\bullet}$, as well as certain oxidizing agents and proradicals, such as ozone O_3, peroxynitrite $ONOO^-$, singlet oxygen $^1O_2$, hydrogen peroxide H_2O_2, nitrogen dioxide radical $NO_2^{\bullet}$, nitrous acid HNO_2, hypochlorite ClO^-, etc. These compounds are formed in mitochondria as a result of aerobic metabolism and are responsible for the correlation between lifespan and age-related diseases. There are other enzymatic ROS sources: peroxysomal xanthine oxidase, NADPH oxidase, etc. Chemical, photochemical, and radiochemical sources of ROS also exist.

It is believed that ROS are involved in the transmission of cellular signals, for example, between mitochondria and cellular nuclei or between cells [10].

At the molecular level, certain cellular components, especially in postmitotic tissues, undergo a very slow and quite an inconsiderable renewal, and. therefore, exist over the whole life of an organism. A damage in structural elements of cellular components, called here critically weak links [6], leads to a decay and complete loss of the function of the components in certain processes (oxidation, hydrolysis, etc.; Fig. 1). Such critically weak links can be located in DNA (damages result in chain breakage and mutations), proteins (decay, change, or loss of functions, disturbance of protease recognition, which is necessary to destruct damaged proteins), and lipids (oxidation affects permeability and "fluidity" of membranes).

In lipids, ROS usually oxidize the methine position of the 1,4-diene fragments [12] (Fig. 2). Certain protein amino acids are oxidized by ROS to hydroxyl and carbonyl derivatives (Fig. 3), and, therewith, proteases are unable to recognize and destroy deeply oxidized proteins [13]. From the quantitative viewpoint, nucleic acids are less prone to oxidation (Fig. 4). However, this oxidative damage [14] deserves particular attention because of the mutagenic conesquences of such damages. The most important type of oxidation in the above examples is that involving C–H bond cleavage. We suggest that the isotope effect can be used to make critically weak links more resistant to ROS damage and thus enhance vitality of the whole organism [11].

Many cellular components susceptible to irreversible chemical changes and associated with aging and age-related diseases, such as oxidation and nitration, belong to the groups of essential or arbitrarily essential components, i.e. they should enter the body

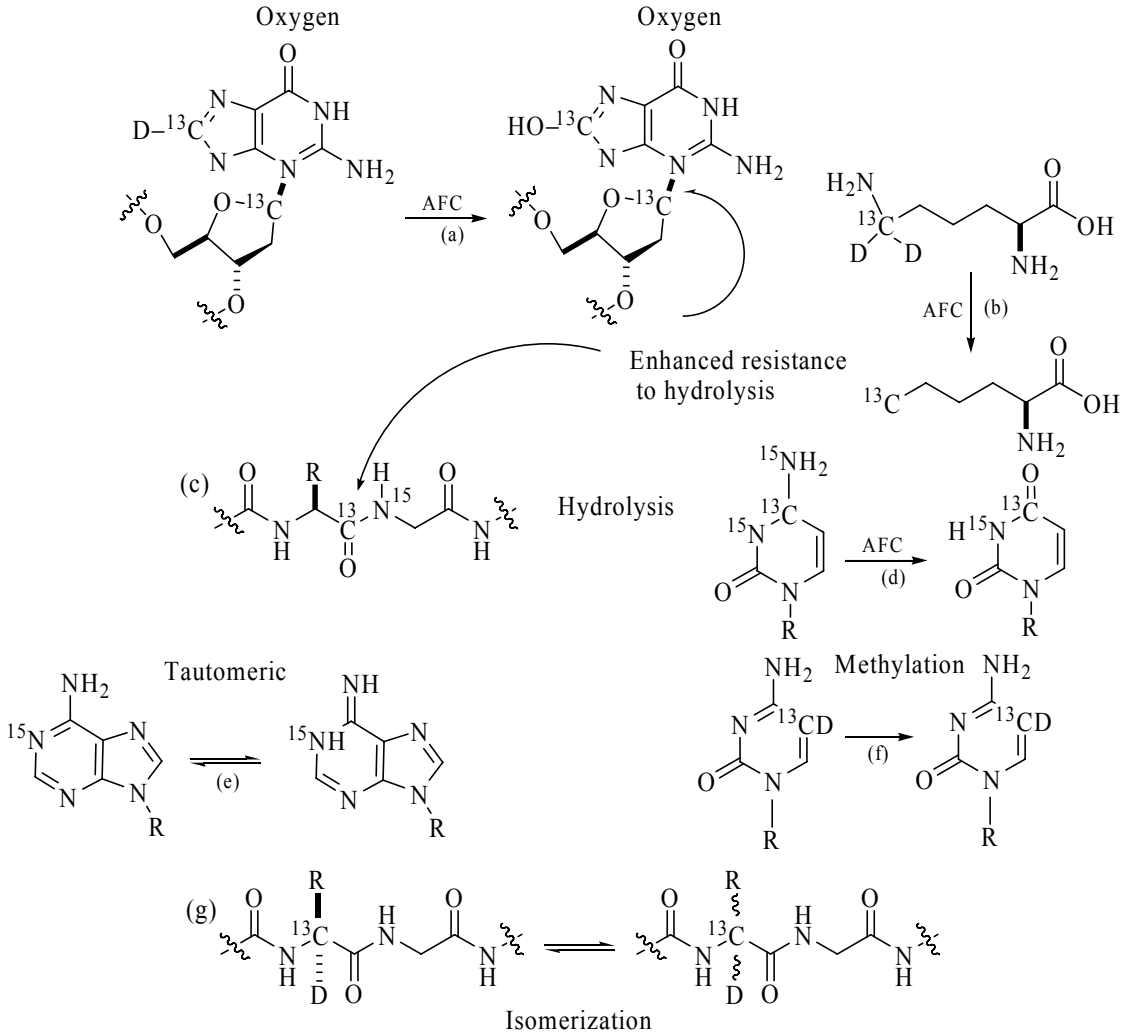

Fig. 1. Example critically weak links which could be «reinforced» by the heavy isotopes ^{13}C and ^{15}N: (a) oxidative transformation of desoxyribonucleotide dG into the mutagenic 8-hydroxy-dG; (b) oxidative carbonylation of lysine; (c) hydrolysis of the peptide bond; (d) mutagenic deamination of cytosine; (E) shift of the tautomeric equilibrium (the shown tautomers are complementary by different bases); (f) methylation of rybonucleotide C (causes epigenetic effects); (g) isomerization–racemization of amino acids (the big arrow points to enhanced stability). The «reinforcement» of polyunsaturated fatty acids to the action of ROS is shown in Fig. 2.

with food.[1] Since only specific atoms/sites of the components under oxidative damage (see Figs. 2–4), then a possibility appears to make use of the isotope effect to protect these sites from oxidation. Therewith, the replacement of atoms by their heavy isotopes does not affect the chemical structure of the components. The intake with food of such isotopically protected components can supply the organism with "building blocks" more resistant to oxidative damage.

In such essential components, the sites susceptible to oxidation (via proton abstraction) can be "reinforced" by replacing hydrogen by deuterium (see Fig. 2c). Simultaneous replacement of two ^{12}C atoms by ^{13}C will further "enhance" this site.

An example of a molecular "time capsule" is DNA whose replication is associated with cell division and results in that DNA only slightly changes its atomic

[1] Essential diet components cannot be synthesized *de novo* and should enter the body with food. Arbitrarily essential components are essential at certain conditions. There are ten essential amino acids for humans: phenylalanine, valine, triptophane, threonine, isoleucine, methyonine, hystidine, leucine, lysine, and, until an age of 5 years, arginine. Purines and pyrimidines are arbitrarily essential components [15]. The essential fatty acids include ω-3 and ω-6, whereas the unsaturated oleic acid is not essential. Essential amino acids, especially arginine and lysine, are much more susceptible oxidation under the action of ROS, than nonessential [13, 16].

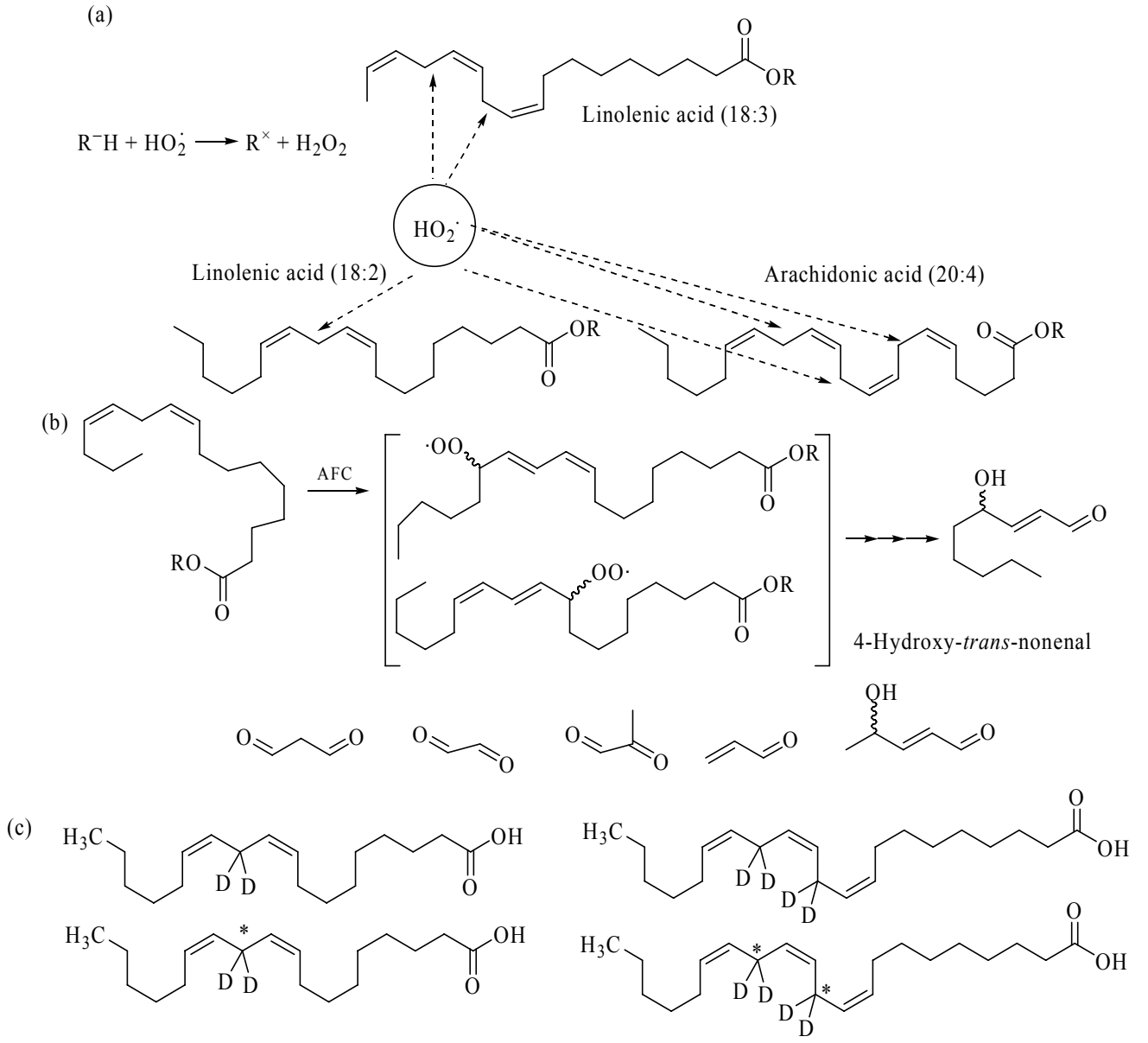

Fig. 2. Schemes of lipid oxidation and protection: (a) sites of the initiation of radical oxidation of certain polyunsaturated fatty acids; (b) certain secondary oxidation products possessing a considerable cellular toxicity; and (c) isotope protection of readily oxidized sites.

and isotopic composition [17] in postmitotic tissues over the whole life of an organism (this was shown by measuring ^{14}C in different tissues before and after birth). The integrity of DNA is necessary over of the whole cell life cycle for correct protein synthesis (it is known that DNA damage is enhaced in genes with a reduced expression level in the aging brain core [18]). In this reasoning one should bear in mind that the postmitotic tissues, such as heart and brain, are of key importance for determining the life expectancy of the whole organism.

It is also suggested that brain cells contain non-DNA components which undergo a slow and an inconsiderable circulation, for example, histones. Neurons circulate through synapses containing proteins which remain unchanged over the whole life cycle [19].

An ideal use of isotopes in biology might be to introduce point changes in critically weak links so that to reinforce molecules by making them more resistant to damages like ROS oxidation or hydrolysis.

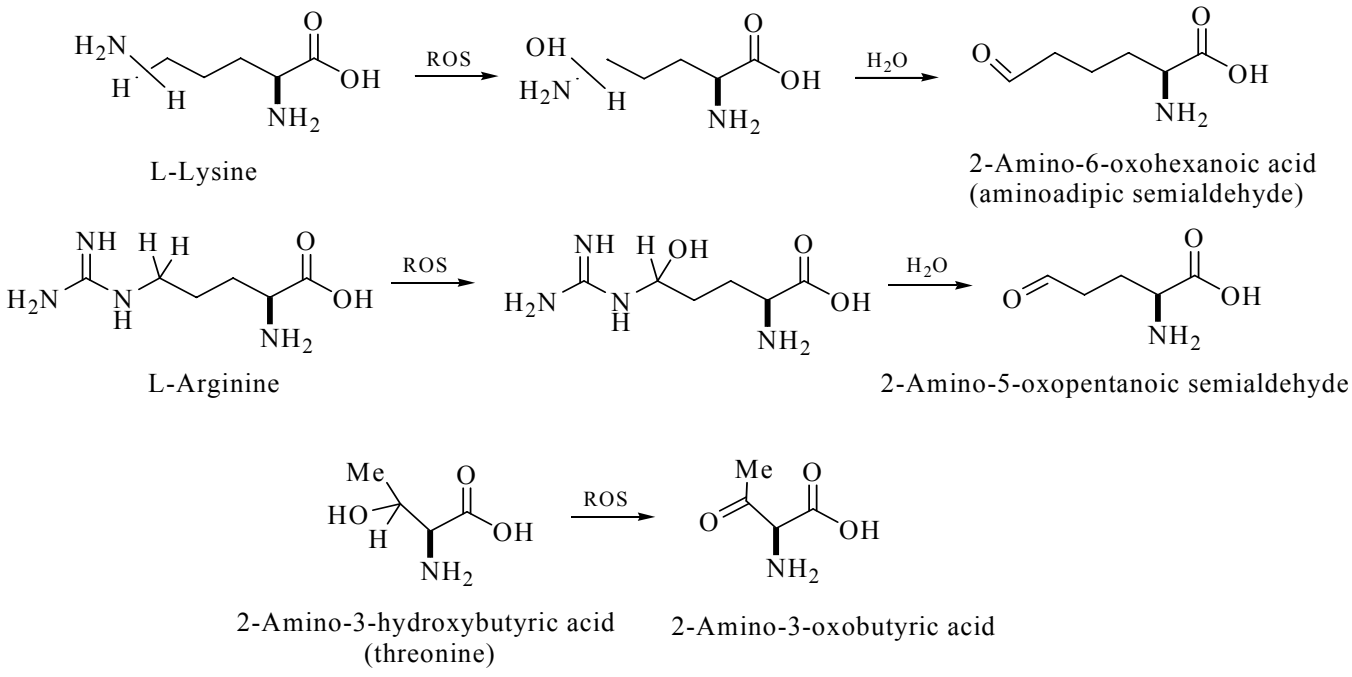

Fig. 3. Scheme of the radical oxidative carbonylation of certain amino acids. Aromatic acids can also be oxidized by ROS (not shown in the scheme).

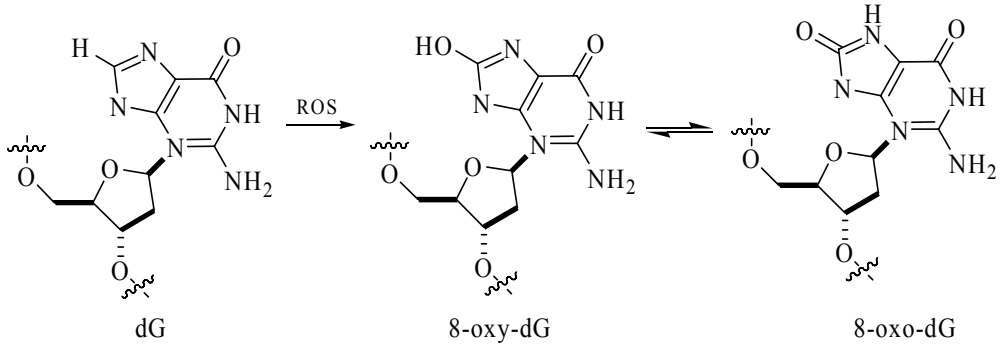

Fig. 4. Example radical oxidation of purine nucleotides.

The ROS-induced oxidative damages in certain amino acids are shown in Fig. 3. It should be stressed that essential amino acids are almost all prone to nonenzymatic oxidation with ROS. The rate-limiting stage in these reactions is hydrogen abstraction. Arginine and lysine are oxidized via successive substitution in these amino acids of ω-hydrogens by hydroxyls [16]. (Like arginine, proline is oxidized to glutamic semialdehyde). Threonine is oxidized to the corresponding oxo acid. Aromatic amino acids are oxidized under the action of ROS to hydroxy derivatives, sometimes with ring opening.

The removal of hydrogen from C_α from the polypeptide chain forms al alkoxyl radical which further forms a hydroxy derivative or peptide chain cleavage via a diamide or α-amidation [16]. Since this hydrogen is fairly mobile, racemization is also possible. It is known that in certain "long-living" proteins, such as crystalline or collagen, the number of D-amino acids increases with age.

Amino acids that contain ^2H and ^{13}C instead of ^1H and ^{12}C should be more resistant to ROS-driven oxidation. To reduce the probablity of abnormal cellular metabolism, the isotope protection should be applied to only those sites in an amino acid, which are susceptible to ROS-driven oxidation. The same relates to components of nucleic acids and lipids. Therefore, the "reinforcement" should involve the ω-atoms (see Figs. 3 and 5) in lysine and arginine.

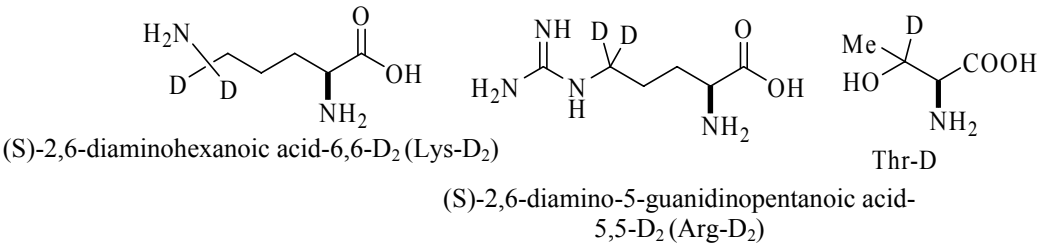

Fig. 5. Protection of amino acids from radical oxidative carbonylation.

The introduction of heavy isotopes in the sites most vulnerable to damage, can enhance resistance of critically weak links resistant not only to oxidation [11] (Fig. 1b), but also to hydrolysis (Fig. 1c) and isomerization–racemization [6, 20, 21] (Fig. 1g). For example, collagen (about 1000 amino acid residues) consists predominantly of proline, oxyproline (25%), and glutamic acid (>30%) and undergoes almost no renewal [22, 23]. As shown, the rates of hydrolysis of the peptide bond in this protein for ^{14}N and ^{15}N differ as little as 0.005, but this results in an enrichment by 15‰ with respect to the first 99% of collagen which hydrolyzes [24] (see Fig. 2c); the collagen proline, oxyproline, and glutamic acid are enriched with ^{15}N. The collagen "lifespan" is normally quite long. Thus, additionally slowing down its degradation by means of the isotope effect may prove quite desirable.

Oxidative damage of nucleic acids occurs not infrequently even under normal physiological conditions. Every day each cell in a body undergoes up to 10^4 such oxidative damages to form diverse abnormal structures in nucleic acid molecules, which cause mutations. An example of an oxidative damage which is particularly important for the mitochondrial function is the formation of 8-oxo-dG. Oxidative damages of DNA accumulate with age in many human body tissues [25].

Other ROS-vulnerable sites in nucleosides/nucleotides, such as the glycoside bond, can be "reinforced" in a similar way. Like with amino acids, deuterated derivatives of nucleic acids are synthesized easier than ^{13}C, but simultaneous ^{2}H and ^{13}C substitution can even stronger enhance resistance to ROS-driven oxidation [11].

Probably, the best advantage of the suggested isotope reinforcement of (bio)molecules as a protection from the stochastic radical oxidation processes can be gained in the case of polyunsaturated fatty acids. This expectation is based not only on high expermental isotope effects reported for these acids [26, 27], but also on the fact that their oxidation plays an important role in cellular degradation. The products of radical oxidation of polyunsaturated fatty acids are in themselves radicals and can initiate further radical oxidation of lipid mambranes, proteins, etc. Moreover, acids are oxidized into various aldehydes, in particular, 4-hydroxy-*trans*-nonenal (see Fig. 2b). These aldehydes, not being radicals, cannot be neutralized by antioxidants and are capable of diffusing in a cell by considerable distances. However, they are quite reactive and form quite a few toxic products. It was shown that aldehydes can irreversibly damage proteins and cause DNA mutations (both by modifying DNA directly and by making it more accessible for ROS by modifying hystons and reparative systems) [28, 29]. A correlation between the accumulation of such damages, aging, and diseases associated with oxidative stress (for example, neurologic) was established.

The key ROS that oxidizes polyunsaturated fatty acids by the chain mechanism is the nonpolar radical $HO_2^{\bullet}$ [12]. The superoxide radical $O_2^{-\bullet}$ several orders of magnitude faster reacts with hydroxides than with lipids. The hydroperoxide radicals $HO_2^{\bullet}$ form inside the mitochondrial lipid biolayer or at the very its surface [30, 31]. The methylene group of the 1,4-diene system is much easier oxidized with ROS than methylene linked to the allyl group (see Fig. 2): Oleic acid is stable under the above-described conditions. The ROS-initiated chain reaction in mitochondrial lipid biolayers disturbs integrity (fluidity) of membranes and damages other macromolecules. A similar oxidative process causes damages in low-density lipoproteins, which contributes much in the development of atherosclerosis and diabetes [32]. Fatty acids can be protected from ROS by "reinforcing" by the isotope effect the methylene groups of the 1,4-butadiene fragments [11] (see Fig. 2c). It is important to understand that site-specific deuteration can hinder the undesirable oxidation of polyunsaturated fatty acids (and, as a consequence, reduce production of toxic carbonyls), therewith not affecting formation of important cellular

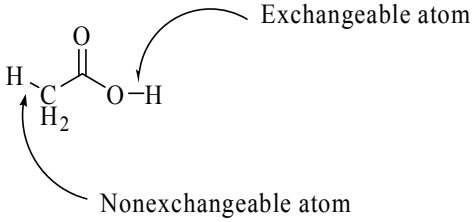

Fig. 6. Exchangeable and nonexchangeable hydrogen atoms in the molecule of acetic acid.

metabolites, such as prostaglandins produced from arachidonic acid.

Heavy isotope substitution does not create the problem of toxicity; toxicity information is available for D_2O only. Heavy water is incompatible with life in mammals at concentrations of above 35% but is nontoxic at lower (<20%) concentrations [33]. It should be mentioned that the effects of heavy water are quite complicated and have not yet studied in detail. Toxicity of deuterated biopolymers, too, is scarcely explored. It is only obvious that substitution in nonexchangeable positions (Fig. 6) should not result in an appreciable release of heavy water and, consequently, such compounds can be expected to be nonhazardous.

It is shown that ^{13}C is nontoxic, which is not surprising. Thus, feeding mice immediately after discontinuation of breastfeeding with a diet in which 80% of carbon was substituted by ^{13}C resulted in that about 60% of carbon in mice gradually substituted by ^{13}C, reaching an equilibrium in 127 days. These mice looked absolutely healthy over the entire experiment. The subsequent necropsy and hystopathologic analysis revealed no appreciable damages which might be caused by isotope substitution. Experiments on mouse embryos, too, revealed no appreciable toxicity [34]. Heavy nitrogen and oxygen isotopes (^{15}N and ^{18}O), too, exhibited no toxicity [35]. Unfortnately, no data are available in the literature on the effect of isotope substitution on lifespan.

The strength of isotope effect in the above-described protected essential diet components can only be determined experimentally. Obviously, the effect should depend on the type of the diet component and on the nature of the oxidative agent. The kinetic isotope effects in the nonenzymatic DNA strand breaking by the hydroxyl radical [36] and nonenzymatic oxidation of fatty acids [26] is close to that in the reaction with alcohol dehydrogenase (k_H/k_D = 2–6).

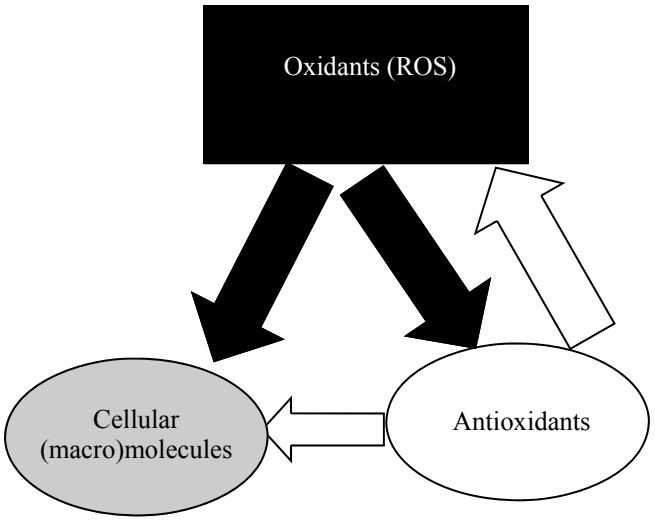

Fig. 7. Simplfied scheme of the reaction of cellular macromolecules, reactive oxygen species, and antioxidants.

With the less reactive HO_2, a more appreciable effect can be expected.

Further evidence for the performance of the suggested approach is provided by experiments with ethanol oxidation with alcohol dehydrogenase. The rate-limiting stage of the oxidation of ethanol to acetaldehyde is hydrogen abstraction. The rates of ethanol oxidation and its deuterated and tritiated analogs were compared. The kinetic isotope effects V_{max}/K_m for the oxidation of (1-R)[1-$^2H_2$]- and (1-R)[1-$^3H_2$]-ethanol with alcohol dehydrogenase to acetaldehyde at pH 6 were 3 and 6.5, respectively [37]. The $D(V_{max}/K_m)$ value obtained in rat in vivo liver studies [38] was 2.89.

Since food components most vulnerable to oxidative damage are classed with essential or ar-bitrarily essential, to supply their isotopically protected forms to an organism seems not quite a challenging problem. To reduce the risk of deceleration of important metabolic/catabolic processes, one should "reinforce" not all but only ROS-sensitive sites in a molecule. To retard the development of age-related cellular accumulation of damaged (oxidized) molecules, the "reinforced" essential components should be introduced in an organism as food additives. Thus introduced "replaceable" isotopically substituted food components will be "diluted" by their *de novo* synthesized analogs, thereby decreasing the kinetic isotope effect.

In future biotechnological processes will be developed for culturing bacteria, yeast, or bacterial strains empoverished with essential components in media

containing the corresponding isotopically protected ingrediaents. By feeding fish, poultry, or cattle with thus produced biomass, one can include the protected essential components in them and thus include these components into the human food chain.

The price of stable isotopes should not pose a barrier and will decline with increasing demand on them. Taking account of the natural abundances of the isotopes (H 99.985% and D 0.015%; ^{12}C 98.89% and ^{13}C 1.11%), we can expect that much more economical techniques for their isolation will be developed, compared with those available in the present time [33].

CONCLUSIONS

On the three types of molecules involved in the oxidative damage process: oxidants, antioxidants, and substrates, the first two were studied in detail to find ways to neutralize oxidants and increase the amount of antioxidants. Until now nobody tried to "reinforce" the target of oxidants (Fig. 7, gray field), specifically macromolecules and other cellular components, which are damaged by ROS.

There are a lot of mechanisms for "repairing" dameged cellular components. However, eventually, the victory is always on the side of the "powers of darkness," i.e. ROS: Aging and diseases kill an organism, even though it resists. This process can be represented as a chemical reaction whose equilibrium is shifetd to an undesirable side. The isotope protection can give a unique chance to affect this reaction: The reduction of the efficiency of oxidation under the action of ROS can slightly shift the equilibrium and thus radically shift it in favor of "positive" processes.

Such compounds (probably, those also containing ^{15}N and ^{18}O) should be synthesized and tested for stability *in vivo*. With ^{13}C (without D), the total isotope effect is lower, but, at the same time, the probability of side effects is also lower.

Attempeted control of oxidative stress with anti-oxidants either have a limited success or, sometimes, an adverse effect. This is associated both with the very nature of antioxidants (capable, for example, of reducing oxygen and converting it into a reactive radical) and with their constant intracellular concentration which, however, in insufficiently high to prevent stochastic damages of biomolecules with radicals. Furthermore, complete removal of ROS from the cellular medium is undesirable, since radicals are involved in cellular signal transmission. Gentle retardation of destructive processes could retard the aging process in itself and prevent age-related diseases. Thus, our method represents a new approach to fighting the oxidative stress and its consequences. The method can find applications in other fields, where there is a demand to slow down certain chemical reactions: from materials science to pharmacology and epienergy [11].

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