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BIOMARKERS OF OH RADICAL DAMAGE *IN VIVO*

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Mechanisms of formation of *o*-tyrosine (*o*-Tyr) and thymine glycol (TG), the two possible markers of OH radical generation in biosystems and *in vivo* are described. The *o*-Tyr measurements require invasive approaches, while TG detection may be accomplished by noninvasive analysis in the urine.

KEY WORDS: Hydroxy radical, ortho-tyrosine, phenylalanine, thymidine glycol, thymine.

INTRODUCTION

Whether generated *via* endogenous or exogenous processes, free radicals in biological systems have been implicated in carcinogenesis, inflammation, toxicology and various other diseases and disorders.[1] Of particular interest have been active oxygen species, such as hydroxy radical, which are generated by Haber-Weiss reaction,[2] ionizing radiations and, presumably, normal and pathological metabolic processes.[3] Since they are extremely reactive with most biocomponents ($k(\cdot OH)$ is on the order of 10^9 - $10^{10} M^{-1} s^{-1}$),[4] the lifetime of hydroxy radicals in biological matrices is extremely short ($< \sim 1$ ns). Hydroxy radicals, therefore, are not readily detectable in complex biological systems by conventional physicochemical techniques. To study OH radical damage *in vivo*, alternatives for monitoring free radical processes must be developed.

Highly specific products of free radical reactions that can be detected in biological matrices are candidates for use as biomarkers of these reactions *in vivo*. Such steady state products are stable over long periods of time (e.g. days, months, or years) and are much more easily measured by traditional analytical methods than are free radicals themselves. As long as other sources of these final products do not exist, they can be used as biomarkers to monitor free radical processes in simple biochemical systems, cells, and even entire organisms.

Biomarkers may come from two classes of reactions involving: (a) endogenous components, such as amino acids and DNA bases, and (b) exogenous substrates, such as spin traps and DMSO, added to cells or organisms. Hence, on the basis of their origins, biomarkers may be classified as either "endogenous" or "exogenous."

In 1948 Stein and Weiss undertook an experiment to demonstrate radiation induced splitting of water into OH radicals, using aqueous solutions of benzene and benzoic acid, and measuring the corresponding radiolytic products. They reasoned that if "hydroxyl radicals are formed primarily, it should be possible to isolate some of the corresponding substances containing OH groups, that is products of hydroxylation. This indeed, proved to be the case."[5] The tendency of hydroxy radical to add to double bonds, and aromatic and heterocyclic rings, suggests hydroxylated biomolecules as possible biomarkers of OH radical reactions. Since many non-radical

metabolic processes also lead to hydroxylations, however, the choice of modified molecules to be monitored is extremely limited.

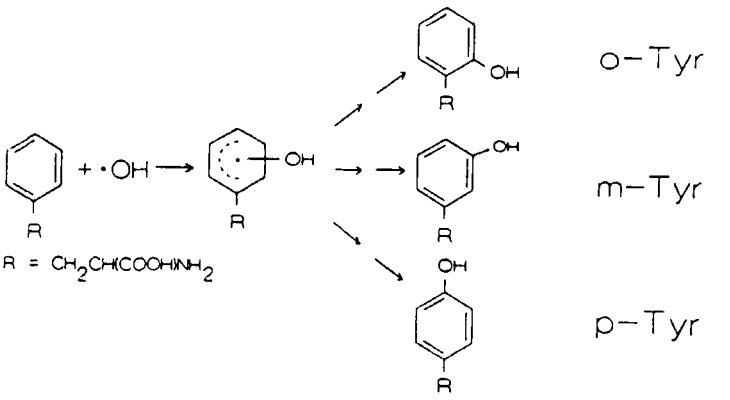
We shall present two examples of endogenous biomarkers of OH radicals (*o*- tyrosine and thymine glycol) and demonstrate how these biomarkers are generated in biosystems when OH radicals are generated within those systems by ionizing radiation.

COMPONENTS OF PROTEINS

The interaction of ·OH with proteins is of special concern because of the importance of proteins in the structure, protection and repair of the damaged biological system. Although, genetically, DNA damage may have more dire consequences for the organism, protein damage (especially of DNA repair enzymes) may exacerbate the damaging effects of free radicals. In addition, the greater concentration of proteins in cellular systems makes them a major target for ·OH.

Because of the propensity of ·OH to add to aromatic rings, the aromatic amino acids phenylalanine, tyrosine and tryptophan are prime candidates in the monitoring of free radical interactions. The susceptibility of tryptophan and its derivatives to destruction by acid hydrolytic techniques[6] greatly restricts the usability of hydroxylated tryptophan as a biomarker. The major product of ·OH addition to tyrosine, 3,4-dihydroxyphenylalanine (3,4 DOPA),[7] is a component found at high background levels in many biological systems and is, therefore, also unsuitable as a marker of ·OH attack on proteins.

Hydroxylation of phenylalanine (~4% of all amino acids) leads to the formation of ortho-, meta- and para-tyrosines.[8,9]



Para-Tyrosine is the amino acid normally incorporated into proteins and would not, therefore, be an appropriate marker of ·OH reactions. Meta- and ortho-tyrosine (*o*-Tyr) are not incorporated into the protein structure, because of the high specificity of tRNA, and could be, therefore, suitable biomarkers.[10] Recently, *in vitro* and under autooxidative conditions, *o*-Tyr has been reported to be formed from the action of isolated biological enzymatic systems (hypoxanthine-xanthine oxidase)[11] and crude tissue homogenates on free phenylalanine.[12] However, recent work has refuted the suggestion of detectable *o*-Tyr formation from hydroxylation of phenylalanine by

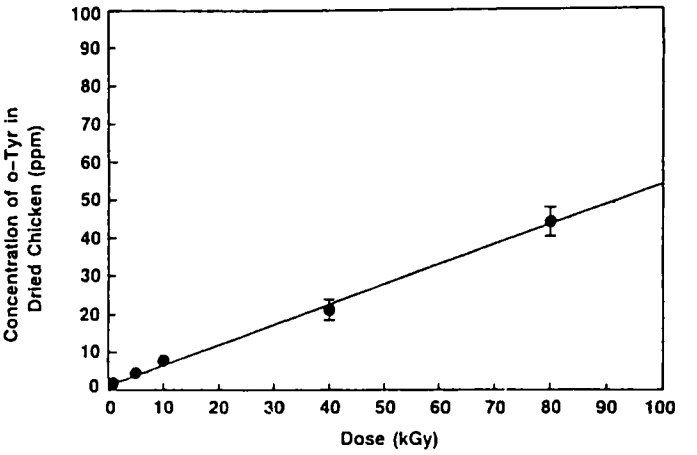


FIGURE 1 Dose yield response of *o*-Tyr formation in irradiated chicken breast meat, water insoluble fraction (fiber). Experimental details appear in reference[16].

enzymatic systems *in vivo* in the absence of free-radical generating agents.[13,14] This would suggest that either the background levels of free ·OH *in vivo* under normal conditions are too low to greatly modify proteins, or that incidental modifications of proteins are efficiently removed and eliminated from the organism. Since *o*-Tyr has not been detected above the 0.1 ppm level in fresh muscle tissue not exposed to radiation or oxidative stress,[10,14,15] its presence above this level could be used as an indicator of ·OH generation *in vivo*.[10,16,17]

Whether ·OH is generated by the effect of ionizing radiation on water or *via* the Haber-Weiss reaction, *o*-Tyr has been shown to be formed by the reaction of phenylalanine OH-adducts with oxygen.[8]

In the absence of oxygen, part of the free radical intermediates (OH-adducts) combine to give biphenyl derivatives.[18] Using radiation as a generator of OH radicals in fresh chicken muscle tissue, *o*-Tyr as measured by gas chromatography/mass spectrometry increases with increasing dose.[10,17] Figure 1 shows a dose-yield plot of *o*-Tyr formation in chicken breast meat water insoluble component (fiber) and authenticates *o*-Tyr as a biomarker for radiation-induced ·OH. Detection of *o*-Tyr in fresh chicken muscle tissue exposed to organic solvents such as carbon tetrachloride, benzene and ethanol also demonstrated OH radical generation.[16,17]

Reactions of OH radicals with aliphatic and sulfur containing amino acids are less specific and will not be discussed in this brief review.

COMPONENTS OF DNA

Aside from adding to aromatic amino acids such as phenylalanine, ·OH also adds to the double bonds of DNA bases. Free radical-induced modification of DNA, if unrepaired or repaired erroneously, has the potential of leading to mutagenic or carcinogenic events or even to cell death. Fortunately, cells and organisms have developed repair systems to correct for and remove inappropriately altered bases by excision and glycolytic repair.[19] When altered bases such as thymine glycol (TG) are

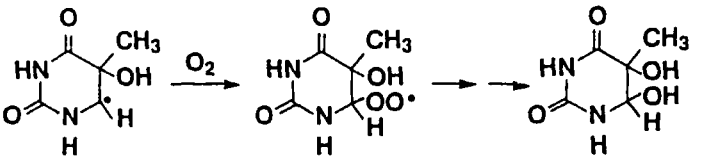
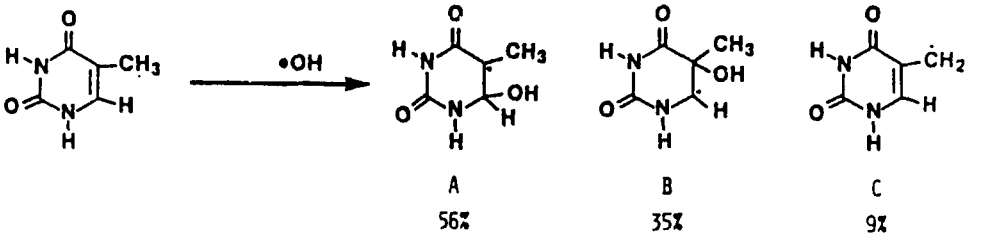


FIGURE 2 Mechanism of hydroxy radical induced thymine glycol formation in the presence of oxygen.

excised from the DNA molecule in mammals, the filtration and purification processes of the body concentrate the excised products in the urine. No biochemical processes have been shown to generate TG, therefore, measuring levels of this and other released products of ·OH attack on DNA bases in the urine could provide a noninvasive detection of *in vivo* free radical processes.[20]

The reaction of ·OH with thymine has been shown to produce three radical intermediates[21]



In the presence of oxygen, radical B reacts with O_2, and subsequently leads to the formation of TG[22] (Figure 2). Thymine glycol will also be formed by interaction of radical A with oxidants and some other radicals without participation of oxygen. No other biochemical processes have been demonstrated to generate TG.

Thymine glycol can be removed from DNA by either glycosylase or excision repair pathways[23] to yield TG, its nucleoside, thymidine glycol (dR-TG), and other products (ΣP). The eventual excretion of these products into the urine can be monitored for non-invasive, indirect measurement of ·OH radical reactivity with thymine in DNA.[24] In order to test TG as a viable urinary biomarker of OH radical generation *in vivo*, whole or parts of living organisms were exposed to ionizing radiation, a well known generator of OH radicals in soft tissues. The levels of urinary dR-TG in irradiated mice and cancer patients are linear with dose or total energy input.[23,24] Data for patients undergoing radiation therapy of cancer are shown in Table I, demonstrating a link between ·OH and TG production *in vivo*.

Since the only known mechanism for the production of TG is the reaction of ·OH with thymine, and since induction of hydroxy radicals *in vivo* and TG production can be correlated, TG seems to be an acceptable biomarker for the presence of ·OH *in*

TABLE I

Yield of dR-TG excreted in the urine of human cancer radiotherapy patients (4) before and after exposure to ionizing radiation (from reference 23).

Dose (Gy)	dR-TG Excreted (nmol/kg per day)	Yield of dR-TG (nmol/kg/day/Gy)
0	0.3 ± 0.1	0.37 ± 0.1
1.8	1 ± 0.3	

vivo. Other hydroxylated DNA bases could also qualify as biomarkers for hydroxy radical reactions *in vivo* by similar reasoning, provided other sources of their generation have been eliminated.

CONCLUSIONS

Thymidine glycol and ortho-tyrosine are two well characterized biomarkers for both non-invasive and invasive measurements of OH radical interactions with DNA and proteins *in vivo*. Other similar compounds may be useful for monitoring as well as for quantifying free radical reactions *in vivo*, however, the possibility of non-radical sources of these substances must be eliminated before these molecules or their moieties can be accepted as biomarkers of free radical reactions with biological systems.

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