Formation of ortho-Tyrosine by Radiation and Organic Solvents in Chicken Tissue*

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Fresh chicken breast and beef incubated in water were found to contain no o-Tyr at the current levels of detection (0.1 ppm) by capillary gas chromatography/mass spectrometry and selective ion monitoring. In contrast, samples incubated at 37 °C in the presence of ethanol, benzene, or carbon tetrachloride (used in fat extraction) contained large quantities (2.5–5.1 ppm) of o-Tyr. No o-Tyr was detected in the water-insoluble fraction of meat treated with carbon tetrachloride after triple extraction by water. However, reaction of radiation generated 'OH in γ-irradiated fresh chicken tissue with endogenous phenylalanine yields o-Tyr with a linear yield-dose response in both water-soluble and -insoluble tissue fractions. Nonradiolytically generated 'OH is suggested to be formed through a mitochondrion-mediated Haber-Weiss reaction in association with water-soluble proteins since the yields of o-Tyr in beef, a tissue with a higher mitochondrial content, are four times greater than in the chicken breast tissue.

Generation of hydroxy radicals in biosystems has been a topic of numerous debates. Major hindrances to 'OH detection have been their extremely short lifetimes due to their high reactivity, $k = 10^6$ to $10^{10}$ M$^{-1}$ s$^{-1}$, with most organic molecules, and the absence of a suitable indicator ("biomarker") of 'OH generation. Certain radiolytic products of aromatic amino acids, however, may qualify as specific markers of hydroxy radical generation. Due to a high proportion of water, ionizing radiation generates large quantities of hydroxy radicals in soft tissue. Therefore, measurement of 'OH induced products in irradiated tissues and their yields may serve to identify samples which have been irradiated and, also, contribute to the understanding of 'OH generation by other mechanisms.

Tyrosine is present in all proteins at about 1–3% levels as para-tyrosine (p-Tyr). It is not an essential amino acid since it is synthesized metabolically from phenylalanine (Phe), e.g.

\[
Phe \xrightarrow{\text{Phe-hydroxylase}} \text{tetrahydropterine} \xrightarrow{p-Tyr} \]

Phe-hydroxylase is a monooxygenase which oxidizes Phe in the 4-position specifically, using tetrahydropterine as a cofactor.

Radiation-generated hydroxy radicals also hydroxylate Phe by addition to the benzene ring (1–3). The reaction, however, is not restricted to the 4-position. Hence, three isomers are generated.

\[
\text{R} = \text{-CH} - \text{CH(NH)COOH}
\]

The Phe-OH intermediates (o-, m-, and p-isomers) react with themselves, other oxidizing radicals, oxidants, and oxygen to give the three isomers of Tyr after subsequent reaction processes. Consequently, all three Tyr isomers are expected in Phe-containing systems which are subject to 'OH generation such as during irradiation in the presence and in the

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In the presence of air at ambient temperature (20 °C), irradiation-Pieces of chicken breast meat (-50 g) were irradiated with a Co-y-source (dose range 0.5-80 kGy at a dose rate of 0.1985 kGy/min) in the presence of air at ambient temperature (20 °C). Irradiation-Pieces of chicken breast meat (-50 g) were irradiated with a Co-y-source (dose range 0.5-80 kGy at a dose rate of 0.1985 kGy/min) in the presence of air at ambient temperature (20 °C).

EXPERIMENTAL PROCEDURES

Materials—Fresh (never frozen) meats (within 24 h of slaughter) were used throughout these studies. Hydrochloric acid and HCl/propionic acid mix for hydrolyses, acetone, and benzene for lipid extractions was from Pierce Chemical Co. Analytical grade carbon tetrachloride (CCL) for lipid extractions was from The Warner-Graham Company, and the scotch whiskey used for incubation purposes was 86 proof (43%). All solvents were used without further purification. para-Hydroxyphenylglycine (p-HPG) was from E. Merck, and the scotch whiskey used for incubation purposes was 86 proof (43%). All solvents were used without further purification.

Irradiation-Pieces of chicken breast meat or beef (-50 g) were irradiated with a Co-y-source (dose range 0.5-80 kGy at a dose rate of 0.1985 kGy/min) in the presence of air at ambient temperature (20 °C).

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RESULTS

Yields of o-Tyr from each run were calculated by using the equation:

\[ Y_R = \frac{k \times A_{o-Tyr} \times \text{moles}_p}{A_{p-HPG} \times \text{mg of dried chicken}} \]

where \( Y_R \) is the number of moles of o-Tyr formed per mg of irradiated and dried chicken, \( k \) is the relative response factor of each o-Tyr fragmentation ion with respect to its corresponding p-HPG ion, \( A_{o-Tyr} \) is the integrated peak area of a given o-Tyr ion, \( A_{p-HPG} \) is the area of the corresponding p-HPG ion, and \( \text{moles}_p \) is the number of moles of p-HPG added (1.00 x 10\(^{-3}\)). The \( Y_R \) calculated for each run of any given sample were then averaged to give \( Y_s \) (the average yield of o-Tyr in the sample). The variation of \( Y_s \) in each sample was between 5 and 7%. To determine the yield of o-Tyr at a given dose, the \( Y_s \) of each chicken sample (at least three, but no more than five) at that dose were averaged and the standard deviation determined. Final values are summarized in Table I.

Regression analysis of sample yields, \( Y_s \) \((n = 15)\), for doses 5 kGy and above (after total depletion of oxygen in the tissue) produced a Student's \( t \) value of 10.00 \((r = 0.941)\), resulting in \( p < 0.001\). Below 5 kGy \((i.e. at 0 \text{ dose and } 1 \text{ kGy; } n = 9)\), regression analysis yielded a \( t \) value of 4.82 \((r = 0.863; p < 0.01)\). Data presented in the table are shown in Fig. 3 which illustrates the dose-yield plot for irradiation doses of 0 \((control)\), 1, 5, 10, 40, and 80 kGy exposed chickens \((\text{Ccl,}-extracted)\) before \((\text{WSF+WIF})\) and after \((\text{WIF})\) washing with water. In certain cases, the error bars denoting the standard deviation fall within the diameter of the circle representing the mean. The radiation experiment has been performed on up to five different chickens over a 2-year period, and, in each case, yields were within 30% of the calculated mean at each dose point. The dose-yield plot of nonextracted chicken for irradiation doses of 0, 0.5, 1, 2.5, 4, and 5 kGy, which is within the regulatory range for food irradiation, has been previously shown \((9)\). To obtain the yield of o-Tyr in milligrams/mg of chicken, the number of moles of o-Tyr is multiplied by 181 x 10\(^{3}\) mg/mole (the molecular weight of tyrosine).

The yield of o-Tyr as a function of dose was distinctly different in samples of irradiated chicken meat extracted by \( \text{Ccl,} \) compared with those in which the water-soluble fraction was removed prior to hydrolysis and analysis (Fig. 3 and Table I). The samples containing both water-soluble and -insoluble components \((\text{WSF+WIF})\) exhibited a large positive intercept, while the water-insoluble component \((\text{WIF})\) itself did not show any measurable amounts \((<0.1 \text{ ppm})\) of o-Tyr in nonirradiated samples. It was concluded, therefore, that \( \text{Ccl,} \) may induce nonradiative generation of o-Tyr, i.e. hydroxy radicals, in the sarcoplasm of meat and not in the muscle fiber fraction \((\text{WIF})\).

Measurements of o-Tyr were also conducted without the \( \text{Ccl,} \) fat extraction procedure. Yields of o-Tyr under those conditions \((9)\) in nonirradiated samples was not measurable as in the case of WIF (Fig. 3). Although earlier studies had suggested the presence of o-Tyr in various animal systems \((including human serum)\), more recent work has failed to confirm the presence of o-Tyr in animal tissues in the absence of radiation or other \( 'OH\)-generating processes \((9-11)\).

If the yield of o-Tyr at doses at and below 5 kGy in the WIF, corrected for the loss of WSF \((\sim25\%\); determined by the difference in dry weight of meat sample before and after extraction with water), is combined with the data for irradiated chicken without fat extraction as obtained at doses <5 kGy \((9)\), the data fall onto the same line. This is not unexpected because the radiation yield of o-Tyr should not be too different in WSF and WIF of meat. Since dry protein is 20% of fresh meats, the radiation induced yield of o-Tyr in fresh meats can be easily calculated. For example, the yield of o-Tyr at 100 krad \((1 \text{ kGy})\) in dry protein fraction is 1.35 ppm.
(without CCl₄ extraction) which corresponds to 0.27 ppm in fresh chicken breast. A correlation of the yield of o-Tyr in CCl₄-treated beef muscle (21.5 ppm in dry protein) indicates that o-Tyr generation by CCl₄ is equivalent to 20 kGy (2 Mrad) which generates 4.5 mmol of o-Tyr/kg.

The basic premise that o-Tyr will be generated on irradiation of fresh meats has been clearly demonstrated to take place at room temperature as a linear function of absorbed dose at an approximate yield of 1.23 ppm/kGy (9) in dried protein (0.25 ppm/kGy in wet meat) at doses below 5 kGy and 0.71 ppm/kGy (0.15 ppm/kGy in wet meat) at doses 5 kGy and above. Such a biphasic response of yields is expected from previous studies in model systems which demonstrate that, in the absence of oxygen, the rate of o-Tyr formation from Phe + ·OH in irradiated aqueous solution is approximately 50% of the amounts formed in the presence of oxygen (6). Since residual oxygen in tissue is expected to be completely consumed at a dose of 5 kGy (3), these results confirm the prediction derived from model systems.

**DISCUSSION**

Nonradiolytic generation of ·OH and the controlling factors are not understood for processes in vivo and relevant mechanisms can be only speculated from model systems at present. Physiological reduction of CCl₄ in the liver has been shown to yield ·COCl, radicals (12) which promote autoxidation of fat. Generation of o-Tyr, however, requires an ·OH, which is not formed on either reduction of CCl₄ or autoxidation of lipids. Superoxide radical, ·O₂⁺, was found to be generated by xanthine/xanthine oxidase (X/Xₐₕ) in biosystems (13), and it has been suggested that ·O₂⁺, through formation of H₂O₂ (catalyzed by superoxide dismutase (SOD)) may lead to generation of hydroxy radicals (14), via Fenton-type reactions (15). Superoxide radical and hydrogen peroxide are also generated by mitochondria (16, 17). Since the mitochondria are the site of cellular O₂ metabolism, formation of ·O₂⁺ is expected to occur at the electron transport chain of perturbed organelles (18). Carbon tetrachloride and other organic solvents are less likely to stimulate the X/Xₐₕ system. Solvents are perturbed. Under such generation conditions, ·OH is not directly associated with ethanol and, therefore, is not scavenged. Alcohol consumption has been correlated with an increased rate of debilitating damage and an increase in lipid oxidation (24). Generation of o-Tyr by liqueur (43% alcohol concentration), although less than by absolute (100%) alcohol (Fig. 4), is high enough to raise concern about potential ·OH-induced damage in organisms. Similarly, nonenzymatic cleavage of proteins by reactive oxygen species has been suggested recently to involve hydroxy radicals (25).

Experimental protocols, e.g. solvent extractions, may induce free radical reactions in biosystems. Hence, product and composition measurements may be affected by the experimental protocol and the conditions to which the sample has been subjected. Consequently, it is very important to have methodologies for various biomarkers, e.g. o-Tyr, which would measure spurious and undesirable oxidative processes in order to validate the applied biochemical analytical procedures. If the tissue is subjected to conditions in which o-Tyr is generated by means other than an artifact of the experimental protocol, then comparison of o-Tyr levels in both water-

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**TABLE II**

**Average yields of o-Tyr**

| Solvent | Chicken |
|---------|---------|
| H₂O     | <0.1    |
| C₂H₅CH₂OH | 2.5    |
| CCl₄    | 5.1     |

Concentration of o-Tyr in solvent-incubated (1 h, 37°C) fresh muscle tissue after drying is expressed as parts/million in dried tissue.

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**FIG. 4.** Yield of o-Tyr in chicken breast meat (measured as parts/million in dried tissue) incubated at 37 °C for 1 h in 25 ml of water (H₂O), 100% ethanol (200 proof), 50% ethanol in water, or 86 proof scotch whiskey. Experimental details appear in the text. Yield of o-Tyr in irradiated (2.5 kGy) chicken breast meat (no CCl₄ extraction) is shown for comparison.
soluble and -insoluble fractions would provide important information about \( ^\cdot \)OH generating processes involved in free radical induced tissue damage. Such information may lead to greater insights to not only the free radical-induced damage, but also to potential inhibitors (26) against and repair of that damage in vivo.

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