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A SENSITIVE CHEMICAL PROCEDURE FOR MONITORING THE RADIOLYTIC OXIDATION OF PROTEINS AT \( \gamma \)-RAY DOSAGES DOWN TO 1 Krad

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Summary: The radiolytic degradation of proteins in oxygenated solution is initiated by the attack of OH radicals at a multiplicity of main-chain and side-chain loci to give a wide variety of oxidized protein sites. A major fraction of these oxidation reactions lead to the introduction of reactive carbonyl groups into the protein covalent structure. With γ-rays the total carbonyl yield correspond to $G(>CO)_t = 1$ for a number of different proteins. Outlined here is a sensitive chemical-spectrophotometric procedure for quantitatively monitoring $G(>CO)_t$ over a dose range of interest in radiation biology (1 to 50 Krad). Data obtained with pepsin, α-chymotrypsin and β-lactoglobulin are discussed.
Radiation-chemical damage to protein in oxygenated solution arises from chemical change initiated by the attack of OH radicals formed in the radiation-induced decomposition of water.\(^1\) For many protein systems, a major over-all chemistry can be generalized in terms of the schematics:

\[
\begin{align*}
H_2O + O_2 & \rightleftharpoons HO_2 + OH \\
PH + OH & \rightarrow \dot{P} + H_2O \\
\dot{P} + O_2 & \rightarrow \dot{PO}_2 \\
\dot{PO}_2 + HO_2 & \rightarrow \text{oxidation products.}
\end{align*}
\]

Because of the complex chemical composition of proteins, the attack of OH radicals via reaction 2 occurs at a multiplicity of main-chain and side-chain loci to give a wide variety of protein free-radical sites as evidenced by product analysis,\(^2\) pulse-radiolysis,\(^3,4\) and spin-trapping\(^5\) techniques. A major fraction of the subsequent oxidation reactions 3, 4 leads to the introduction of carbonyl groups (\(>\text{C}=\text{O}\)) into the protein co-valent structure.\(^2\) Carbonyl groups are produced in the radiolytic oxidation of (1) the peptide main-chain to yield \(\alpha\)-keto acid functions \(\text{RCOCONHCHR}_2\) (2) the aliphatic side-chain groups of serine, threonine, lysine, and arginine to yield \(\text{RCHO}\) and \(\text{R}_2\text{CO}\) carbonyls and (3) the unsaturated side-chains particularly tryptophan which yields a carbonyl of the type \(\text{ArCOR}\). The detailed mechanisms of the various radiation induced reactions at main-chain and side-chain loci of peptides, polypeptides and proteins are the subject of a recent monograph.\(^6\)

In previous studies we have shown that the combined yield of carbonyl groups formed at these various sites can be determined by a
chemical-spectrophotometric procedure that involves the formation of
the 2,4-dinitrophenyl hydrazone derivatives. The phenylhydrazone de-
rivatives of most monocarbonyls show essentially the same absorption
spectrum in basic solution and have very similar extinction coeffi-
cients at the wave length of maximum absorption with $\lambda_{\text{max}} = 440 \text{ mu}$,
$E_{440} = 2.4 \times 10^4$. Carbonyl analysis of oxygen-saturated solutions of
pepsin, $\alpha$-chymotrypsin and $\beta$-lactoglobulin after irradiation with $^{60}\text{Co}$
$\gamma$-rays were found to give total carbonyl yields, $G(>\text{CO})_t$ of 1.20, 0.89,
and 1.25 respectively over the dose range 50–500 Krad.\textsuperscript{2} These yield
values account for a major fraction of the OH radicals formed in step 1
with $G(\text{OH}) = 2.8$.

We now find that this spectrophotometric procedure, under certain
specific conditions, can be adapted to quantitatively monitor the ra-
diolytic oxidation of aqueous proteins at $\gamma$-ray dosages as low as 1
krad (and lower under optimum experimental conditions). In a typical
"low-dose" procedure, an aqueous pepsin solution (0.5%, pH 3) is equi-
librated with air or oxygen and irradiated in a sealed pyrex vial (10
ml) with $^{60}\text{Co}$ $\gamma$-rays from a 200 curie source at a dose rate of
$\sim 1$ krad/min. Immediately after irradiation, the solution is dialyzed
in cellophane against water until hydrogen peroxide cannot be detected
with titanium sulfate reagent. The dialyzed protein solution is then
acidified to 0.1N with hydrochloric acid, treated with 2,4-dinitro-
phenylnhydrazine (2,4 DNPH) reagent to give a five to ten-fold excess
of reagent over the estimated carbonyl concentration and allowed to
stand at room temperature (25°C) for 4 hours to effect the quantitative
formation of the hydrazone derivatives. Higher 2,4-DNPH concentra-
tions, longer reaction times, and reaction temperatures above - 25°C favor a "dark-reaction" between protein carboxyl groups and 2,4-DNPH which yields interfering chromophoric protein-hydrazones.\(^2\) The reaction mixture is then dialyzed in cellophane against water at 25°C for a period of 15 to 18 hours to quantitatively remove excess reagent. Aliquots of the dialyzed solution are made 1.5N in potassium hydroxide to develop the anion chromophores of the protein hydrazones and the absorption spectra are immediately recorded over the 350 to 600 m\(\mu\) range using a Cory spectrophotometer Model 14, with a 1 cm or longer path. Control samples of the unirradiated protein solutions are carried through the entire procedure in parallel with the irradiated samples; the calculations of carbonyl yields are based on the differential spectra.

Figure 1 compares relative spectra of the hydrazone derivatives of irradiated pepsin with hydrazones derived from a number of monocarbonyls of the type RCOCONHCHR\(_2\), R\(_2\)CO, RCHO and ArCOR.\(^2,7-9\) Very similar spectra are obtained with the hydrazone derived from irradiated \(\alpha\)-chymotrypsin, \(\beta\)-lactoglobulin and soluble collagen (gelatin).

Figure 2 shows the observed optical densities of the hydrazone anions derived from irradiated pepsin as a function of dose under the experimental conditions described here. The control corrections for unirradiated pepsin, \(\alpha\)-chymotrypsin and \(\beta\)-lactoglobulin are uniformly low. At dosages of 5 Krad and above the corrected optical densities of the irradiated samples are reproducible to \(\pm\) 2 percent. At 1 Krad the optical density of the control amounts to \(~\) 20 percent of the irradiated value and the reproducibility at this dose level is within \(\pm\) 6 percent.
The experimental procedure described here provides us, for the first time, with a technique for quantitatively determining major products formed in the radiolysis of proteins in oxygenated solution at biologically relevant dosages down to at least 1 krad. We are presently using this low-dose technique in studies of the radiolytic oxidation of aqueous histone and nucleohistone with the objective of adapting the procedure to permit a quantitative study of the radiolytic oxidation of chromosomal proteins in-vivo.
References

FIGURE CAPTIONS

Fig. 1. Relative absorption spectra of 2,4-dinitrophenylhydrazone derivatives of (1) irradiated pepsin — (2) RCHO(acetaldehyde) — — — — (3) RCONHCHR₂(pyruvoylalanine) — — — — (4) R₂CO(acetone) — — — (5) ArCOR(acetylphenone) — — — — [in 1.5N KOH]

Fig. 2. Optical density of irradiated pepsin hydrazones — ● — — —, and unirradiated control, — ○ — — [in 1.5N KOH].
Fig. 1
Fig. 2

Optical density

Dose in krad
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