

# Immunochemical Detection of Oxidized Proteins<sup>1</sup>

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An immunochemical assay was developed to detect carbonyl moieties that result from oxidative damage to proteins. Bovine serum albumin was reacted with hydroxyl radicals generated via a Fenton-like mechanism or by a radiolysis mechanism. The resulting albumin-derived carbonyls were reacted with 2,4-dinitrophenylhydrazine, giving the corresponding hydrazones, which were detected by Western blot using anti-dinitrophenyl antisera. The immunoblot demonstrated a concentration-dependent increase in carbonyl formation, as well as fragmentation of the albumin into two distinct bands with molecular masses of 51 and 45 kDa when oxidized with the Fenton-like mechanism, and 62 and 46 kDa when oxidized by radiolysis. Analysis of the immunoblot using laser densitometry indicated a linear relationship between carbonyl groups and increasing treatment from radiolysis. This immunochemical assay was approximately 3 orders of magnitude more sensitive than the spectrophotometric method and was able to determine the molecular mass of carbonyl-modified polypeptides in the detection of oxidative damage.

## Introduction

Proteins are one of the cellular components vulnerable to damage by oxidants, and increased carbonyl content in proteins from aldehyde and ketone formation is an indication of oxidative stress. Oxidative stress is associated with a shift in the prooxidant and antioxidant balance in favor of the prooxidant. This may occur as a result of increased oxygen tension, increased metabolic rates, a decrease in the levels of normal cellular antioxidants, or exposure to redox-active exogenous agents (1). Oxidative damage to proteins has been shown to result in increased protein turnover and decreased enzymatic function and has been associated with a number of pathological processes, including emphysema, atherosclerosis, and neurological diseases (2). Studies in the laboratories of Stadtman (3) and Davies (4) have demonstrated that protein oxidation is a normal physiological event that marks proteins for degradation by proteolytic systems, but levels of oxidized proteins are increased during conditions of oxidative stress.

The laboratory of Davies published a series of papers on protein damage and degradation by oxygen radicals (5-7). These studies documented many changes that occur to proteins as a result of oxygen radical exposure, including decreases in native fluorescence, shifts in isoelectric point, and both increases and decreases in molecular weight due to either covalent interactions or peptide cleavage. They demonstrated that oxidative damage to proteins in red blood cells is an early event, occurs independently of membrane damage, and is a more sensitive indicator of oxidant damage than is lipid peroxidation (8).

There are currently no techniques available to analyze the individual oxidized proteins in a complex mixture of

oxidized and nonoxidized proteins. The current methods of assessing protein oxidation denature the proteins, precluding any possibility for identification of the individual proteins that are oxidized. There are active research efforts to refine methods for the determination of lipid peroxidation and DNA damage (9-12), and the development of a sensitive measure of protein oxidation using immunochemical methods will be a major addition to the area of oxidative damage.

The purpose of this study is to develop an immunochemical assay to detect oxidative protein damage, to validate the assay by direct comparison with the established spectrophotometric techniques, and to utilize the technique to assess oxidative damage to bovine serum albumin.

## Experimental Procedures

**Chemicals.** 2,4-Dinitrophenylhydrazine was purchased from Fluka (Buchs, Switzerland). Vanadyl sulfate was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Bovine serum albumin (A 4503) and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO). Vanadyl stock solutions were prepared to 5.0 mM using a molar extinction coefficient at 750 nm of 18.0 M<sup>-1</sup> cm<sup>-1</sup> and were adjusted to pH 2.0 to prevent air oxidation (13). Concentrations of hydrogen peroxide were estimated using a molar extinction coefficient of 43.6 M<sup>-1</sup> cm<sup>-1</sup> at 240 nm (14). All reagents used were reagent grade and were prepared daily in distilled-deionized water.

**Oxygen Radical Generation. (A) Metal-Catalyzed Reactions.** Bovine serum albumin (BSA)<sup>2</sup> was oxidized using a hydroxyl radical generating system consisting of vanadyl and hydrogen peroxide. The vanadium system has been demonstrated to rapidly yield significant quantities of hydroxyl radical at pH 7.4 (15-19). BSA has been used as a model protein in many oxygen radical studies (4). Each sample (1.0-mL total volume) contained 1 mg of BSA, 1 mM H<sub>2</sub>O<sub>2</sub>, and the indicated amount of vanadyl sulfate in 10 mM sodium phosphate buffer (pH 7.4). Proteins were derivatized with 2,4-dinitrophenylhydrazine immediately following the addition of vanadyl.

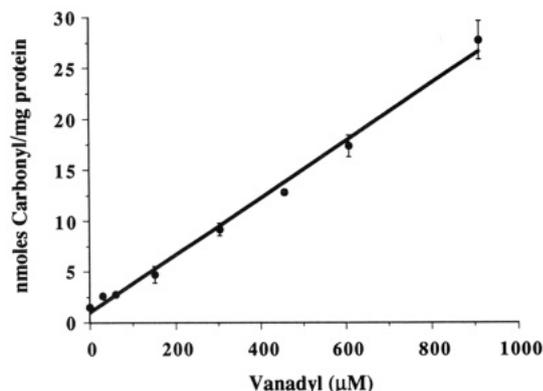
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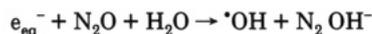
<sup>1</sup>An abstract of this work has been presented at the 1993 annual meeting of the Society of Toxicology in New Orleans, LA (*Toxicologist* 13, 374).

<sup>2</sup>Abbreviations: BSA, bovine serum albumin; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis.



**Figure 1.** Spectrophotometric determination of carbonyl derivatives generated by the reaction of BSA with vanadyl and hydrogen peroxide. Each sample (1.0-mL total volume) contained 1 mg of BSA, 1 mM  $H_2O_2$ , and the indicated amount of vanadyl sulfate in 10 mM sodium phosphate buffer (pH 7.4). Each point represents the mean  $\pm$  standard deviation of six experiments.

**(B) Radiolysis.** BSA (1 mg/mL) was prepared in double-distilled and deionized water, saturated with 100%  $N_2O$ , and irradiated with different doses of  $\gamma$ -radiation using a J. L. Shepard and Associates Model 143 cesium-137 irradiator (Glendale, CA) at a dose rate of 1.385 Gy/min. Saturating the solution with  $N_2O$  results in approximately 99% of the total radical generation being hydroxyl radicals (5) due to the following reaction:

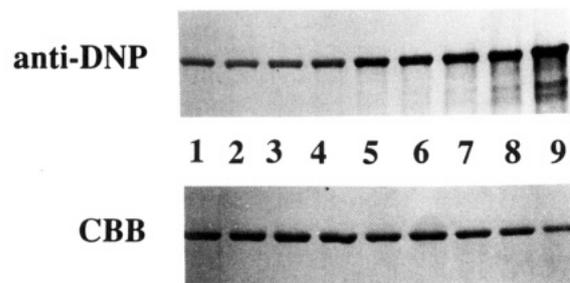


**Carbonyl Determination. (A) Spectrophotometric.** Carbonyl content was determined spectrophotometrically according to the method outlined by Levine et al. (20). Oxidized proteins were reacted with 2,4-dinitrophenylhydrazine in the following manner. To approximately 1 mg of protein was added an equal volume of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl, and the mixture was allowed to stand at room temperature for 1 h. The reaction was terminated with the addition of 20% trichloroacetic acid, the samples were centrifuged at 11000g, and the supernatant was discarded. The pellets were washed three times in ethanol/ethyl acetate (1:1), and the precipitated protein was redissolved in 6 M guanidine solution at 37 °C. The carbonyl content was calculated from the maximum absorbance at 367 nm using a molar extinction coefficient of 22 000  $M^{-1} cm^{-1}$ . Spectrophotometric measurements were performed using a Perkin Elmer Lambda 6 UV/vis spectrophotometer.

**(B) Immunochemical.** Oxidized BSA was reacted with an equal volume of 0.5 mM 2,4-dinitrophenylhydrazine in 0.1 M sodium phosphate buffer (pH 6.3), incubated 1 h at room temperature, and separated electrophoretically under reducing conditions with sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE)<sup>2</sup> (21). Proteins were transferred electrophoretically (22) to nitrocellulose, which was incubated at room temperature for 2 h with anti-dinitrophenyl antisera (Dakopatts, Denmark; diluted 1:1000) using previously published methods (23), followed by a 1.5-h incubation with mouse anti-rabbit IgG conjugated with alkaline phosphatase (Jackson Laboratories, West Grove, PA). The membranes were then developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in 0.1 M Tris-HCl buffer (Bio-Rad substrate kit, Bio-Rad Laboratories, Richmond, CA). Nitrocellulose membranes were scanned with a Molecular Dynamics computing laser densitometer (Sunnyvale, CA), and the relative intensities were determined using Image Quant data analysis system.

## Results

The results of the spectrophotometric determination of vanadyl-catalyzed protein oxidation are presented in Figure 1, indicating that carbonyl groups are formed in a



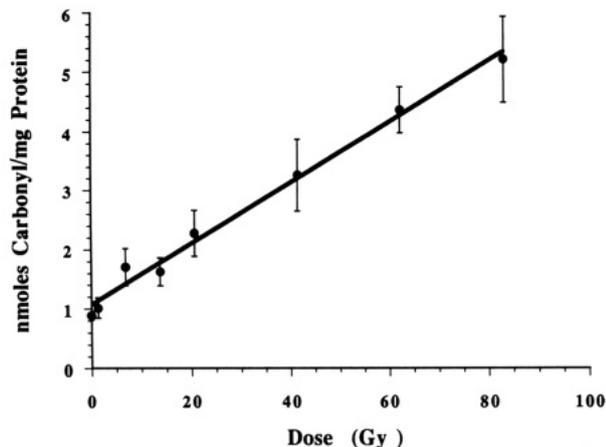
**Figure 2.** Carbonyl groups on BSA derivatized with 2,4-dinitrophenylhydrazine and detected by immunoblot using anti-dinitrophenyl antisera (upper panel, 0.75  $\mu g$ /lane). Protein content was determined by staining with Coomassie blue (lower panel, 2  $\mu g$ /lane). Protein was oxidized by metal-catalyzed oxidation using vanadyl as a catalyst in the following concentrations: lane 1, 0 mM (no  $H_2O_2$ ); lane 2, 0 mM; lane 3, 0.03 mM; lane 4, 0.06 mM; lane 5, 0.15 mM; lane 6, 0.3 mM; lane 7, 0.45 mM; lane 8, 0.6 mM; lane 9, 0.9 mM.

concentration-dependent manner from the reaction of vanadyl and hydrogen peroxide in the presence of BSA.

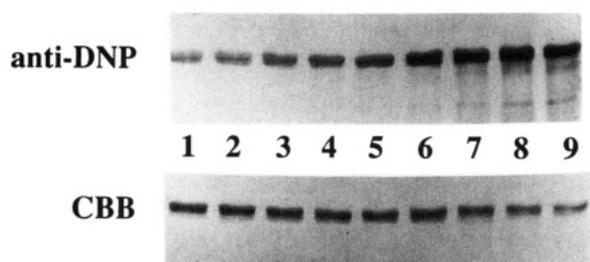
To determine if the carbonyl groups could be detected with immunochemical methods, BSA was oxidized by a Fenton-like reaction of vanadyl and hydrogen peroxide under identical conditions to those reported in Figure 1 using the indicated amounts of vanadyl sulfate to produce a concentration dependent increase in the oxidation of BSA. BSA was initially separated by SDS/PAGE. One gel was stained with Coomassie blue (Figure 2, lower panel), the other gel was transferred to nitrocellulose, and the oxidized proteins were immunochemically detected using polyclonal rabbit anti-dinitrophenyl antisera following previously published methods (23).

In Figure 2 (lower panel) there is a decrease in the intensity of the albumin stained with Coomassie blue. This is consistent with oxidative damage such as aggregation and fragmentation and has been reported by Davies and Delsignore (6) in irradiated proteins, and in proteins treated with copper(II) and hydrogen peroxide by Marx and Chevion (24). The upper panel is the immunoblot detecting the oxidized proteins using anti-dinitrophenyl antisera. There is a concentration-dependent increase in the metal-catalyzed carbonyl formation on albumin. In Figure 2 there are two major fragments of approximately 51 and 45 kDa that also contain carbonyl groups indicated by the staining with the anti-dinitrophenyl antisera. The results obtained in Figure 2 use 1300 times less protein than those in Figure 1, demonstrating the sensitivity of the immunochemical technique.

Figure 3 shows the results of the irradiation experiments. BSA was irradiated as described in the Experimental Procedures, and the carbonyl groups were determined spectrophotometrically. Carbonyl groups were formed in a linear fashion with increased dose of radiation. Figure 4 shows the immunochemical results from BSA irradiated in an identical manner to the samples in Figure 3. The lower panel is again consistent with radiation-induced fragmentation described by Davies and Delsignore (6). Albumin oxidized by radiation contains two major fragments that contain carbonyl groups (Figure 4, lanes 6-9) that increase with increasing radiation. The relative molecular masses of the fragments are 46 kDa, similar to the 45 kDa seen in Figure 2, and a 62-kDa fragment. The specificity of the immunoblotting analysis was confirmed by showing that the immunochemical reactions could be inhibited by 2,4-dinitrophenol (Figure 5).



**Figure 3.** Spectrophotometric determination of carbonyl derivatives generated by radiolysis. Each sample contained 1 mg of BSA saturated with nitrous oxide in 1.0-mL total volume. Each point represents the mean  $\pm$  standard deviation of four experiments.



**Figure 4.** Carbonyl groups on BSA derivatized with 2,4-dinitrophenylhydrazine and detected by immunoblot using anti-dinitrophenyl antisera (upper panel, 2  $\mu$ g/lane). Protein content was determined by staining with Coomassie blue (lower panel, 2  $\mu$ g/lane). Protein was oxidized by radiolysis in the following amounts: lane 1, 0 Gy; lane 2, 1.39 Gy; lane 3, 6.93 Gy; lane 4, 13.85 Gy; lane 5, 20.78 Gy; lane 6, 27.70 Gy; lane 7, 41.55 Gy; lane 8, 62.33 Gy; lane 9, 83.10 Gy.

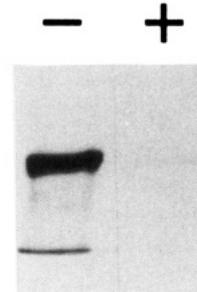
Quantitation of the staining intensity was determined by transmittance densitometry of the blot (Figure 6). The intensity was determined by volume integration of the bands corrected for background and shows a linear increase with radiation dose.

### Discussion

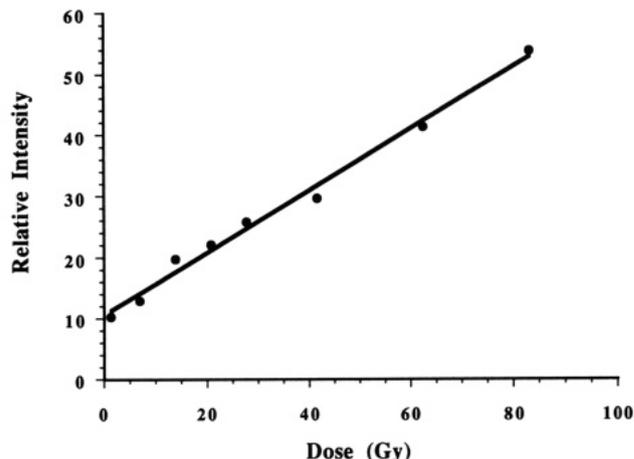
These results demonstrate a new technique which is applicable for immunochemical determination of protein oxidation. The technique has been shown to determine carbonyl groups formed by oxidation of protein by either metal-catalyzed or radiolytically generated oxygen radicals. A comparison of the technique with the spectrophotometric assay indicates that oxidative damage can be detected immunochemically using 3 orders of magnitude less protein.

Previous methods for the determination of carbonyl content in oxidatively modified proteins primarily involve four techniques, and the methodology, advantages, and disadvantages of each technique have been discussed by Levine et al. (20). The primary techniques available are as follows: (a) protein carbonyl groups are reacted with 2,4-dinitrophenylhydrazine to form stable protein hydrazones which are detectable spectrophotometrically; (b) reduction of the carbonyl group with tritiated borohydride to a radiolabeled alcohol; (c) fluorescence measurements of the hydrazones produced by reaction with fluorescein

### 2,4-Dinitrophenol



**Figure 5.** Inhibition of immunoblot detection of carbonyl groups generated by radiolysis on albumin by 2,4-dinitrophenol. Albumin treated with 42 Gy was separated by SDS/PAGE (2  $\mu$ g/lane), transferred to nitrocellulose, and probed with anti-dinitrophenyl antisera (1:1000 dilution) that had been incubated at 4  $^{\circ}$ C overnight in the absence (-) or presence (+) of 16 mM 2,4-dinitrophenol.



**Figure 6.** Densitometer determination of carbonyl groups following immunoblot using anti-dinitrophenyl antisera.

hydrazide; and (d) spectrophotometric measurements of stable secondary amines produced by reaction of the carbonyl groups with fluoresceinamine to form Schiff bases followed by reduction of the Schiff base with NaCNBH<sub>3</sub>. The reaction of carbonyl groups with tritiated borohydride is currently the most sensitive method available for the determination of oxidized proteins. This technique has been modified to determine carbonyl groups formed in vivo (25), but cannot detect individual oxidized proteins, and the use and disposal of radioactive compounds limit the usefulness of the technique.

The most commonly used method to determine carbonyl content of proteins is spectrophotometric detection following the reaction with 2,4-dinitrophenylhydrazine, although it is relatively insensitive and requires considerably more sample than the borohydride method. The applicability of the technique in vivo has been hampered because there are no extraction techniques available to separate derivatized proteins from complex mixtures found in tissue homogenates. Preliminary studies in our laboratory indicate that our technique is applicable in vivo, as numerous protein bands stained positive for carbonyl groups following a hepatotoxic dose of acetaminophen.<sup>3</sup>

An immunochemical method to detect oxidized proteins has several advantages over the current methods. One advantage is the sensitivity of the immunochemical assay;

<sup>3</sup>Pumford, Keller, Halmes, and Hinson. Unpublished observations.

it can detect oxidized protein derivatives in the picogram range. Another important advantage of an immunochemical method is the potential to distinguish an oxidized protein in a complex mixture of proteins or in vivo studies. Proteins oxidized by chemicals or radiation and then derivatized by 2,4-dinitrophenylhydrazine can be separated by using SDS/PAGE and then transferred to nitrocellulose and stained with the specific antibody to the 2,4-dinitrophenyl group. Therefore, the oxidation of proteins and their fragments with different molecular weights can be followed, as can determination of the sites on proteins or the proteins most susceptible to oxidative damage.

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