# Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins

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**Metal-catalyzed oxidation results in loss of function and structural alteration of proteins. The oxidative process affects a variety of side amino acid groups, some of which are converted to carbonyl compounds. Spectrophotometric measurement of these moieties, after their reaction with 2,4-dinitrophenylhydrazine, is a simple, accurate technique that has been widely used to reveal increased levels of protein carbonyls in aging and disease. We have initiated studies aimed at elucidating the chemical nature of protein carbonyls. Methods based on gas chromatography/mass spectrometry with isotopic dilution were developed for the quantitation of glutamic and aminoadipic semialdehydes after their reduction to hydroxyaminovaleric and hydroxyaminocaproic acids. Analysis of model proteins oxidized *in vitro* by $Cu^{2+}$/ascorbate revealed that these two compounds constitute the majority of protein carbonyls generated. Glutamic and aminoadipic semialdehydes were also detected in rat liver proteins, where they constitute ≈60% of the total protein carbonyl value. Aminoadipic semialdehyde was also measured in protein extracts from HeLa cells, and its level increased as a consequence of oxidative stress to cell cultures. These results indicate that glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins, and that this reaction is a major route leading to the generation of protein carbonyls in biological samples.**

There is ample evidence to support the notion that the most important mechanism of oxidative damage to proteins is metal-catalyzed oxidation (MCO) (1, 2). This process involves generation of $H_2O_2$ and reduction of Fe (III) or Cu (II) by a suitable electron donor like NADH, NADPH, ascorbate, mercaptanes, etc.; Fe (II) and Cu (I) ions bind to specific metal binding sites on proteins and react with $H_2O_2$ to generate $^{\bullet}OH$. This highly reactive free radical attacks neighboring amino acid residues, some of which are converted to carbonyl-containing derivatives (1, 2). MCO of proteins has been modeled *in vitro* by using a variety of electron donors, and often results in loss of enzymatic activity and alteration of protein structure (3, 4). Carbonyl derivatives can be conveniently measured by sensitive methods, particularly those using 2,4-dinitrophenylhydrazine (DNP), which reacts with carbonyl groups to generate dinitrophenylhydrazones with characteristic absorbance maxima at 360–390 nm (1, 2, 5). By using these methods, it has been established that carbonyl derivatives accumulate on tissue proteins during aging (6–8) and disease development. Increased levels of protein carbonyls are associated with Alzheimer's disease (9), cataractogenesis (10), progeria and Werner's syndrome (6), amyotrophic lateral sclerosis (11), and respiratory distress syndrome (12), among others. Although the experimental evidence is so far mostly correlative, it lends strong support to the hypothesis that the protein carbonyl content of tissues reflects the fraction of oxidatively damaged protein with impaired function, and might therefore be at the root of disease and aging related functional losses (1, 2). It is of note that a number of independent laboratories have measured protein carbonyls in a variety of human and animal tissues and reported values in the vicinity of ≈1–2 nmol/mg protein; given the fact that carbonyl compounds are just a fraction of oxidized amino acids (histidine, tryptophan, methionine, and phenylalanine residues are respectively oxidized to oxo-histidine and aspartate, kynurenines, methionine sulfoxide, and ortho- and metatyrosine, all noncarbonyl products), carbonyl levels represent an underestimation of the extent of oxidative damage sustained by tissue proteins. Thus, one can estimate that the fraction of damaged proteins could be as high as 30% of the total in old animals (7), making MCO a very relevant process *in vivo*. This line of reasoning has been criticized in one study that presented data purporting to show that DNP-based carbonyl assays are plagued with artifacts that make them unsuitable to measure the actual levels of protein carbonyls present in tissue samples (13). Such levels, measured with a modified version of the general method that supposedly eliminates the artifacts, were reported to be exceedingly low, at ≈0.06 nmol/mg protein in rat liver extract (13). In this context, it is of great interest to elucidate the chemical nature of protein carbonyls, not only to better understand the chemical mechanisms leading to their generation, but also to have independent analytical methods to assess their levels in oxidized proteins and in tissue samples. Based on considerations of susceptibility to oxidation of particular amino acids and amino acid homopolymers (14), we focused our studies on glutamic and aminoadipic semialdehydes (Fig. 1), the main products of MCO of polyarginine and polyproline (glutamic semialdehyde) and polylysine (aminoadipic semialdehyde). We present here evidence showing that these two products are the main carbonyl products of MCO of proteins, and that they are present in biological samples.

## Materials and Methods

**Synthesis and Calibration of 5-Hydroxy-2-Aminovaleric Acid (HAVA), d5-HAVA, 6-Hydroxy-2-Aminocaproic Acid (HACA), and d4-HACA.** Unless otherwise specified, all reagents were from Aldrich or Sigma, of the highest purity available. HAVA was synthesized by the method of Swallow and Abraham (15), except that glutamic acid-5-methyl ester, instead of the ethyl ester, was used as the starting material. The product was purified on a Dowex-50W ion exchange column (acid form) followed by precipitation from methanol. For the preparation of d5-HAVA, 100 mg of L-glutamic-2,3,3,4,4,-d5 acid, min. 98% d (Isotec) were converted to the corresponding methyl ester by treatment with methanolic/

Abbreviations: MCO, metal-catalyzed oxidation; DNP, 2,4-dinitrophenylhydrazine; GS, glutamine synthetase; HAVA, hydroxyaminovaleric acid; HACA, hydroxyaminocaproic acid; OPA, ortho-phthalaldehyde; TFA, trifluoroacetic anhydride; TFAME, *N,O*-trifluoroacetyl methyl ester.

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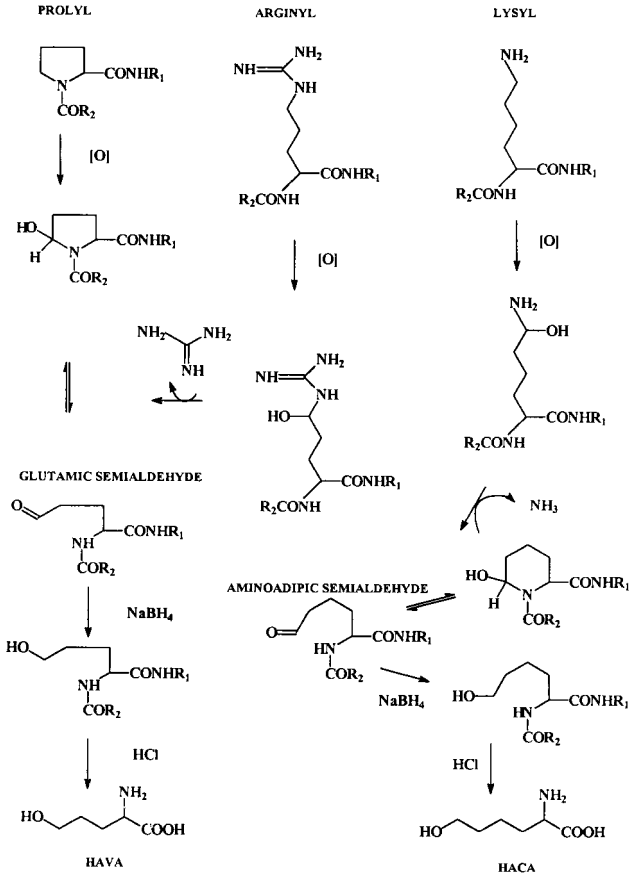


**Fig. 1.** Reaction scheme for the formation of glutamic and aminoadipic semialdehydes and their reduction products HAVA and HACA.

HCl. After evaporation, the product was treated as described for the preparation of HAVA, in a reduced scale reaction. HACA was prepared according to Gaudry (16) and precipitated from methanol. This simple method could not be easily adapted to the preparation of deuterated HACA, given the commercial availability of deuterated precursors; therefore, a modification of the procedure of Baldwin *et al.* (17) was used to prepare d4-HACA. First, 250 mg of D-L-lysine-4,4,5,5-d4.2HCl, min. 98% d (Isotec) were converted to *N*-$\alpha$-formyl-d4-lysine (18). The product was evaporated to an oil and dissolved in 20 ml of water. After adjusting the pH to 9.5 with $NH_4OH$, sodium nitroprusside (320 mg) was added in portions over a 20-min period, while heating at 60°C and adjusting the pH as needed. The resulting brown solution was heated for 4 additional hours, with pH adjustment. The reaction product was then filtered and hydrolyzed in 6 M HCl for 1 h at 100°C. After evaporation, the product was dissolved in dilute formic acid, pH 2, and desalted over Dowex-50W. The final product was precipitated from a small volume of warm methanol. Preparations of HAVA, d5-HAVA, HACA, and d4-HACA were dissolved in deionized water and calibrated against a standard consisting of a mixture of amino acids (Pierce). Samples and standard were analyzed by RP-HPLC after automated ortho-phthalaldehyde (OPA) derivatization using a Hewlett–Packard series 1100 HPLC system. The mean area of Ser, His, Gly, Thr, Arg, Ala, Tyr, Met, and Val was used to calculate the concentration of HAVA, d5-HAVA, HACA, or d4-HACA in each respective preparation. Triplicate analyses were performed for each solution, which was subsequently aliquoted and frozen at −70°C.

**MCO of Model Proteins.** *Escherichia coli* glutamine synthetase (GS) was prepared as described (19). BSA, ribonuclease A from bovine pancreas (RNase), and lysozyme from egg white were obtained from Sigma. Proteins were dissolved at 10 mg/ml in oxidation buffer (50 mM Hepes buffer, pH 7.4, containing 100 mM KCl and 10 mM $MgCl_2$), and dialyzed against the same buffer at 4°C to remove any chelators that might be present in the commercial preparations, or, in the case of GS, as part of the storage buffer. Oxidation was accomplished by supplementing 750 $\mu$l of protein solution ($\approx$7.5 $\mu$g) with a freshly prepared mixture of neutral ascorbic acid and $FeCl_3$ to final concentrations of 25 mM and 100 $\mu$M, respectively, and incubating overnight at 37°C in a shaking bath. Oxidation was terminated by addition of EDTA to 1 mM, and samples were dialyzed at 4°C against oxidation buffer supplemented with 1 mM EDTA. Control samples were prepared in oxidation buffer supplemented with 1 mM EDTA. Protein concentrations were determined with the bicinchoninic acid method (Pierce). Protein carbonyls were measured by an HPLC version of the DNP method, as described (5).

**Biological Samples.** Fisher 344 rat liver proteins were obtained from rat liver homogenates from a rat tissue bank kept at −70°C in our laboratory. The homogenates were prepared by treating minced liver in a tissue homogenizer, extruding the slurry through cheesecloth, and freezing in the form of pellets. For the present studies, equal samples from five animals aged 12 mo were pooled, thawed, and homogenized in PBS containing 1 mM EDTA. Homogenates were centrifuged at 16,000 × *g* for 15 min, and clear supernatants dialyzed at 4°C against PBS containing 1 mM EDTA. HeLa cells were grown in 90% (vol/vol) DMEM supplemented with 10% (vol/vol) FBS; after confluency, cultures were challenged by addition of glucose oxidase and glucose for 6 h, under conditions that resulted in generation of $\approx$1 or 2 mM $H_2O_2$, as measured by using the PeroXOquant peroxide assay (Pierce). After harvesting and washing, cells were pelleted at 210 × *g* for 5 min. The pellet was disrupted by three cycles of freezing and thawing in the presence of protease inhibitors, leupeptin, aprotinin, and pepstatin, 1 $\mu$g/ml each, and 1 mM PMSF, followed by sonication in PBS and vigorous pipetting. Samples were then centrifuged at 16,000 × *g* for 15 min and the clear supernatant collected and dialyzed at 4°C against PBS containing 1 mM EDTA.

**GC/MS Analyses.** Protein samples (10–500 $\mu$g) were reduced with 100 mM $NaBH_4$ in 250 $\mu$l of 250 mM borate buffer, pH 9.2. Reduced samples were evaporated to dryness. After adding a fixed amount of deuterated internal standards (d5-HAVA and d4-HACA), samples were hydrolyzed in 6 M HCl (Pierce) at 155°C in Teflon-lined screw cap vials for 30 min. Hydrolysates were evaporated to dryness, rehydrated in 1 ml of 1% trifluoroacetic acid (TFA), and applied to a Sep-Pak C18 cartridge (Waters) equilibrated in the same solvent. The cartridge was eluted with 1 additional ml of 1% TFA, and the 2-ml eluates dried in a Savant Speed-Vac centrifugal evaporator. Amino acids in the dry samples were converted to their *N*,*O*-trifluoroacetyl methyl esters (TFAME) by sequential treatment with methanolic/HCl and trifluoroacetic anhydride as previously described (20). Samples were then dissolved in 100 $\mu$l ethyl acetate and 1 $\mu$l automatically injected for GC/MS analysis. GC/MS was performed on a Hewlett–Packard model 5890 gas chromatograph equipped with a model 5971A mass spectrometer. An HP-5MS crosslinked 5% phenyl, methyl siloxane column (Hewlett–Packard), was used. The temperature program was 5 min at 60°C, ramp to 190°C at 7°C/min, ramp to 300°C at 15°C/min, and hold at 300°C for 5 min. The injection port was maintained at 275°C. Analytes were detected by selected ion-monitoring GC-MS (SIM-GC/MS). Ions with $m/z$ = 280, 285,

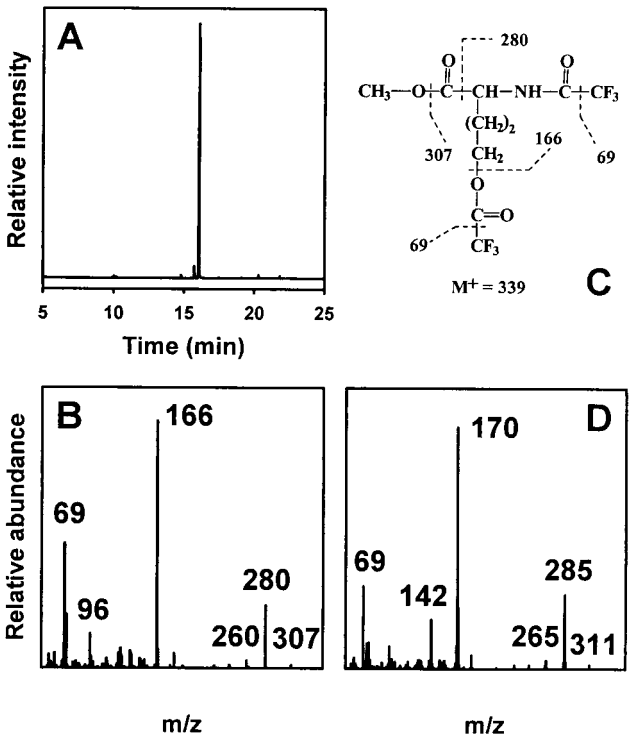


**Fig. 2.** Full scan chromatogram (*A*), mass spectrum (*B*), and fragmentation pattern of HAVA-TFAME derivative. (*D*) Mass spectrum of d5-HAVA-TFAME derivative.

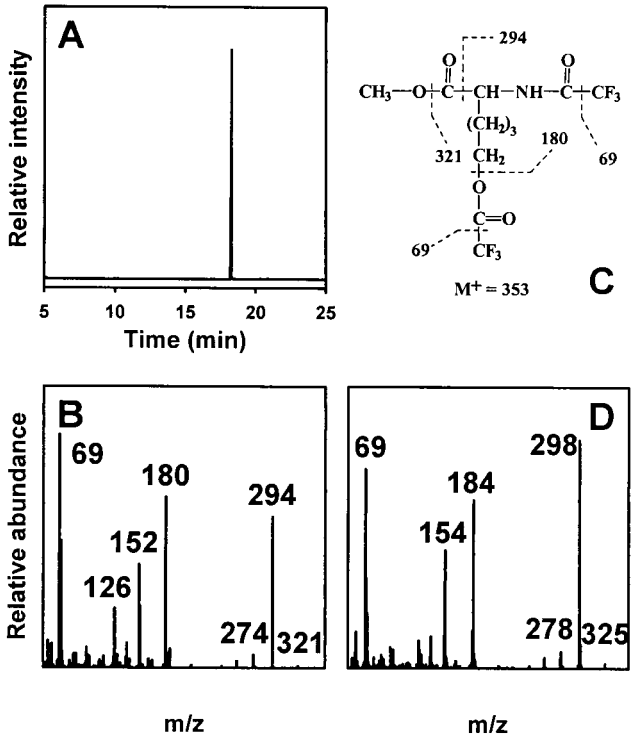


**Fig. 3.** Full scan chromatogram (*A*), mass spectrum (*B*), and fragmentation pattern of HACA-TFAME derivative. (*D*) Mass spectrum of d4-HAVA-TFAME derivative.

294, and 298 were used for HAVA, d5-HAVA, HACA, and d4-HACA, respectively. For each analysis, an external standard curve was constructed by analyzing a series of standards containing the same fixed amount of d5-HAVA and d4-HACA and increasing amounts of HAVA and HACA. These standards were hydrolyzed and derivatized under identical conditions as samples.

## Results

**Design of an Analytical Method for the Measurement of Glutamic and Aminoadipic Semialdehydes.** Glutamic and aminoadipic semialdehydes present in proteins are destroyed by acid hydrolysis, and therefore need to be stabilized by conversion to their corresponding hydroxyamino acids, HAVA and HACA. These two compounds were synthesized according to published procedures, to be used as standards. The identity of HAVA and HACA was confirmed by mass spectral analysis. Full scan GC/MS of the preparations, after TFAME derivatization, yielded chromatograms containing basically one single peak in each case (Figs. 2*A* and 3*A*). The spectra of these peaks correspond to HAVA-TFAME and HACA-TFAME, with characteristic prominent peaks at 280 and 294, respectively, corresponding to the loss of [$COOCH_3$], and 166, 180, after the loss of an additional [$CF_3COO$] [H] (Figs. 2 *B* and *C* and 3 *B* and *C*). These fragmentation patterns are characteristic of TFAME derivatives of structurally related compounds (21, 22).

Deuterated standards, d5-HAVA and d4-HACA produced the expected spectra with peaks showing $m/z$ values shifted to the corresponding higher values (Figs. 2*D* and 3*D*). Preparation of these standards was limited by the necessity of using small amounts of the expensive deuterated starting reagents, which imposed small scale work; the final products were less pure, containing remnants of unreacted starting reagents (d4-lysine, d5-glutamic acid methyl ester, etc.). No attempt was made to further purify d5-HAVA and d4-HACA, as the impurities did not interfere with calibration or GC/MS analyses. HPLC analysis allowed calibration of HAVA, HACA, d5-HAVA, and d4-HACA preparations. Analysis of HAVA and HACA preparations (Fig. 4) showed the presence of two major peaks corresponding to nonstandard amino acids, eluting at positions immediately posterior to arginine (HAVA) and tyrosine (HACA).

For the analysis of HAVA and HACA in protein samples by SIM-GC/MS, ions with $m/z$ = 280, 285 and 294, 298, respectively, were chosen (Fig. 5). Ions 166, 260 and 180, 278, were sometimes used as confirmatory signals. We confirmed the known partial conversion of HAVA to chloro-aminovaleric acid (14) as well as the similar partial conversion of HACA to chloro-aminocaproic acid. These compounds, as well as their corresponding deuterated counterparts, eluted at slightly retarded positions, and their fragmentation patterns (data not shown) showed the characteristic doublets corresponding to the presence of the two main isotopes of chlorine. The intraassay coefficient of variation for glutamic semialdehyde was of 5.19% at a level of 305.27 mmol/mol protein ($n$ = 9), and 8.02% at a level of 4.46 mmol/mol protein ($n$ = 8). For analysis of aminoadipic semialdehyde, these coefficients were of 5.64% at a level of 30.64 mmol/mol ($n$ = 9) and 13.50% at a level of 7.48 mmol/mol ($n$ = 8).

**Glutamic and Aminoadipic Semialdehyde Generation During MCO of Model Proteins.** Small amounts of glutamic and aminoadipic semialdehydes were detected in all native proteins analyzed, namely, GS, BSA, RNase, and lysozyme (Table 1). On MCO, the amount of glutamic semialdehyde increased ≈7- to 14-fold in every case. Aminoadipic semialdehyde levels also increased, albeit in a more moderate fashion. In general, the amount of

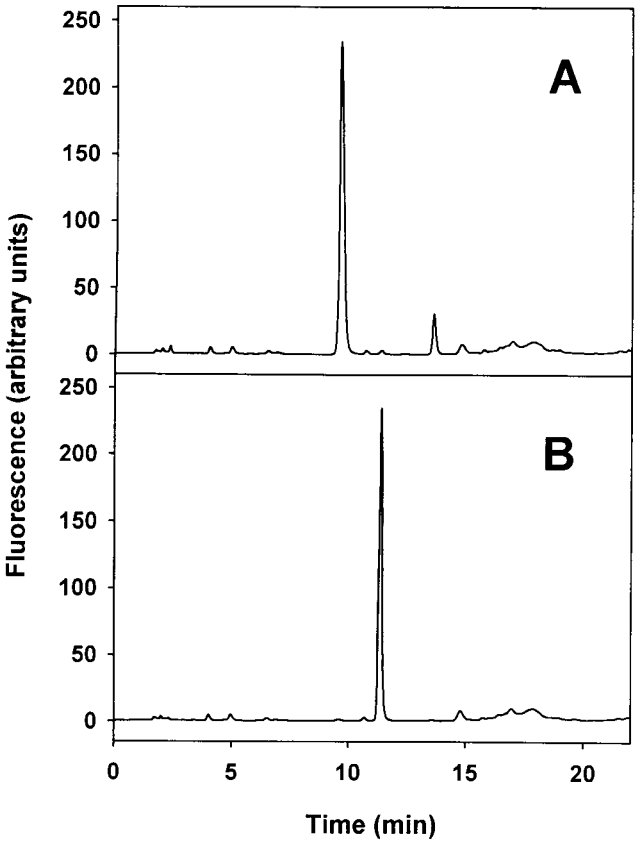


**Fig. 4.** Chromatograms of HAVA (*A*) and HACA (*B*) standards, prepared as described in *Materials and Methods*. OPA derivatives of samples were prepared automatically and derivatized samples analyzed by RP-HPLC.

glutamic semialdehyde in oxidized proteins was higher than that of aminoadipic semialdehyde, with the exception of oxidized BSA, which contains roughly similar amounts of both products, in agreement with a previous study (23). The differences in the yield of both oxidation products from protein to protein partially reflect differences in molecular weight; thus, if values are recalculated and expressed per mg of protein, the disparities are reduced. For example, there are 5.90, 1.91, 1.05, and 1.69 nmol/mg of glutamic semialdehyde in oxidized GS, BSA, RNase, and lysozyme, respectively. It is clear, however, that the disparities also reflect differences in intrinsic susceptibility to MCO under the conditions used. Carbonyl values increased, as expected, after protein oxidation. Carbonyls in native RNase and lysozyme were below the detection limit of our method, and relatively low levels were detected after oxidation, consistent with a lower susceptibility of these proteins to oxidation.

**Glutamic and Aminoadipic Semialdehydes in Biological Samples.** Rat liver proteins contain roughly similar levels of glutamic and aminoadipic semialdehydes (Table 1) Together, these semialdehydes accounted for ≈60% of the protein carbonyl groups measured in the same samples. Glutamic semialdehyde (≈5 mmol/mol) was also detected in proteins from HeLa cells (Fig. 6). This level, lower than that seen in rat liver protein samples, was increased 2.5-fold by treatment of the HeLa cells with the $H_2O_2$ generating system glucose oxidase/glucose. Aminoadipic semialdehyde was below the detection limit of our method in these samples. Given the amount of protein analyzed, its concentration was established to be <1.6 mmol/mol.

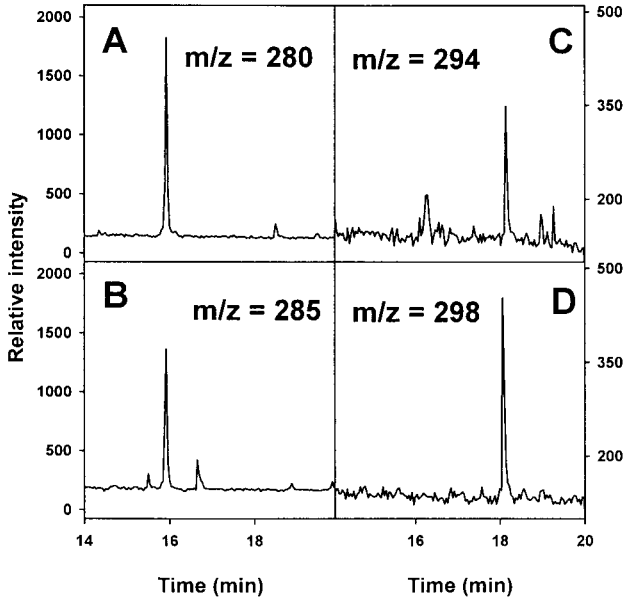


**Fig. 5.** Analysis of HAVA and HACA in an oxidized GS sample. The sample was reduced, spiked with d5-HAVA and d4-HACA, hydrolyzed, and derivatized as described in *Material and Methods*. SIM traces with $m/z$ = 280, 285, 294, and 298 correspond to HAVA, d5-HAVA spike, HACA, and d4-HACA spike, respectively.

## Discussion

There is growing evidence of the involvement of protein oxidation in aging and disease, and measurement of protein carbonyl groups has become one of the most widely used tools to assess protein oxidative damage. Based on previous work (14), we hypothesized that glutamic and aminoadipic semialdehydes would be major products of MCO of proteins. We developed a highly specific isotope dilution SIM-GC/MS method to quantitate these two products in protein hydrolysates after their conversion to HAVA and HACA. The use of deuterated internal standards is a particularly important feature of our method because both HAVA and HACA are partially converted to chloro-derivatives during hydrolysis, whereas HAVA is also partially converted to proline. In a previous study by Ayala and Cutler (24) on glutamic semialdehyde, these difficulties were addressed by a strict control of hydrolysis time and temperature, which should decrease variability in the final yield of analyte; it was also reasoned that treating and analyzing samples and external standards under exactly the same conditions allow a reasonably good way of assuring that factors affecting

**Table 1. Levels of protein carbonyls and glutamic and aminoadipic semialdehydes in model proteins**

| Protein | Carbonyls, mmol/mol | Glutamic semialdehyde, mmol/mol | Aminoadipic semialdehyde, mmol/mol |
|---|---|---|---|
| GS | 14 ± 3 | 21.7 ± 1.2 | 4.3 ± 1.2 |
| Oxidized GS | 359 ± 8 | 305.3 ± 15.8 | 30.6 ± 1.7 |
| BSA | 30 ± 5 | 15.5 ± 0.6 | 11.0 ± 3.6 |
| Oxidized BSA | 398 ± 76 | 126.4 ± 21.9 | 77.8 ± 18.7 |
| RNase | ND | 2.0 ± 0.8 | 1.3 ± 0.3 |
| Oxidized RNase | 18 ± 1 | 14.4 ± 1.0 | 6.0 ± 0.5 |
| Lysozyme | ND | 3.4 ± 0.4 | 3.9 ± 0.9 |
| Oxidized lysozyme | 21 ± 3 | 24.7 ± 5.0 | 4.9 ± 1.3 |
| Rat liver proteins | 42 ± 10 | 10.3 ± 1.0 | 15.6 ± 2.8 |

Values are means from at least three analyses ± SD. Values for rat liver proteins were calculated considering a mean molecular weight of 50,000.

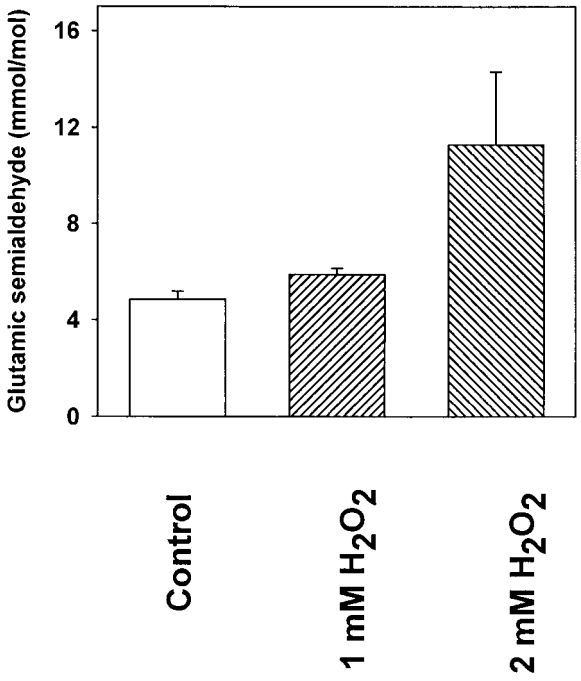


**Fig. 6.** Glutamic semialdehyde in HeLa cells subjected to oxidative stress by exposure to glucose oxidase/glucose. Cells were grown to confluency and then glucose oxidase/glucose added to the medium to generate the indicated concentration of $H_2O_2$ as determined analytically. Results are expressed as mmol/mol of an average 50-kDa protein. Each bar represents analysis of three independent protein samples from a single culture. Error bars are SD.

recovery of the analyte are taken into account (24). However, using deuterated internal standards represents a superior analytical approach, providing a more reliable buffer of variation, not only during the critical hydrolysis step, but also during sample work-up and derivatization. Another important feature of the method described here is calibration of standards against a commercially available primary standard. In contrast, no details on calibration are provided in the aforementioned report (24). These methodological differences most likely account for discrepancies in results. More difficult to reconcile with our findings are the relatively high values of HAVA reported by Ayala and Cutler (24) in some instances in which protein samples that had been oxidized *in vitro* were not reduced before hydrolysis, which prompted these authors to suggest direct formation of HAVA during protein oxidation. In our hands, failure to reduce resulted in total absence of HAVA in sample hydrolysates.

Our analysis of model proteins shows that the majority of carbonyl groups in proteins before and after MCO are glutamic and, to a lesser extent, aminoadipic semialdehydes. In fact, the combined values of these two specific products were in some cases slightly higher than the value obtained for protein carbonyls in the same sample. A similar result has been reported by Pietzsch (25) for his analyses of oxidized low density lipoprotein, and may reflect a slight underestimation of carbonyl groups, a slight overestimation of glutamic and aminoadipic semialdehydes, or both. However, there is an overall remarkable agreement between the two sets of analytical results. Oxidized BSA represents an exception in that its combined levels of glutamic and adipic semialdehydes account only for slightly over 50% of the carbonyl value. This means that, for oxidized BSA, there is a sizeable fraction of protein carbonyls that are not glutamic or aminoadipic semialdehyde. Such is also the case for rat liver proteins, with the two aldehyde products making up ≈60% of the protein carbonyls. The remaining portion of protein carbonyls in these samples might be made up by a product or products of oxidation of amino acid side chains others than arginine, proline, or lysine. In the case of rat liver samples, we should also expect a contribution from carbonyl-containing adducts generated through reaction of proteins with sugars (glycation) and lipid peroxidation products such as 4-hydroxynonenal and malondialdehyde (1, 2). An alternative possibility is the presence of crosslinks as the result of glutamic and aminoadipic semialdehydes reacting with neighboring lysine ε-amino groups to form Schiff bases. Such structures would react with DNP and be accounted for as carbonyls, but, on reduction with $NaBH_4$, they would yield stable secondary amines that would not reverse to HAVA and HACA during acid hydrolysis, thus being "missed." Ongoing studies are being conducted to explore all these possibilities. Oxidized BSA represents a very useful model in such endeavor; of note, whereas data reported here were obtained with BSA that had not been delipidated, parallel studies showed that MCO of lipid-free BSA resulted in similar levels of carbonylation (data not shown).

Our results are also solid arguments refuting Cao and Cutler's (13) criticisms on the validity of carbonyl measurements. Our measurements of glutamic and aminoadipic semialdehydes in rat liver extracts, obtained by using highly specific, well-calibrated methods, show that there are at least 25 mmol of carbonyls per mol of protein, which corresponds to ≈0.5 nmol/mg protein, accounted for as the sum of the two specific carbonyl products measured. Even if one dismisses our carbonyl measurements, and considers this figure not as a minimum but as the total amount of carbonyls of proven existence, this value is eight times the number of "real carbonyls" reported by Cao and Cutler (0.06 ± 0.04 nmol/mg) using a version of the DNP-based procedure modified to supposedly eliminate artifacts. These authors concluded that such extremely low values could not be measured with sufficient reliability. Such conclusion would certainly be reasonable if the reported carbonyl values were correct, however, they are not only incompatible with our results, but also with other data from Cutler's laboratory concluding that glutamic semialdehyde alone accounts for ≈0.15–0.2 nmol/mg protein in liver proteins from mice and humans, i.e., values three to four times their carbonyl measurements (26).

In summary, our results indicate that glutamic and aminoadipic semialdehydes are major carbonyl products in proteins subjected to MCO, in good agreement with the known susceptibility of proline, arginine, and lysine residues to oxidation leading to the formation of carbonyl-containing residues. These two products are also major constituents of the pool of carbonyl products in rat liver extracts, where they make up ≈60% of the total. Measurement of glutamic and aminoadipic semialdehydes in a tissue protein during aging and in association with disease should provide useful information complementing and refining the knowledge that has been gathered using carbonyl measurements.

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