Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5611–5615, June 1992
Biochemistry

Selective cleavage of thioether linkage in proteins modified with 4-hydroxynonenal

(covalent modification of proteins/lipid peroxidation/cysteine residues/Raney nickel)

KOJI UCHIDA AND E. R. STADTMAN*

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Contributed by E. R. Stadtman, March 20, 1992

ABSTRACT **The peroxidation of polyunsaturated fatty acids leads to numerous products, including 4-hydroxynonenal (HNE). That 4-hydroxy-2-alkenal compounds react with sulfhydryl groups of proteins to form thioether adducts possessing a carbonyl function has been established [Schauenstein, E. & Esterbauer, H. (1979) *Ciba Found. Symp.* 67, 225–244]. Taking advantage of the fact that Raney nickel catalyzes cleavage of thioether bonds, we have developed a procedure to quantitate the amount of HNE moiety bound to protein by means of a thioether linkage. Adducts of HNE with *N*-acetylcysteine and glutathione were prepared, labeled with NaB[^{3}H]H$_4$, and then treated with Raney nickel. The ^{3}H-labeled product was recovered in 85–90% yield from both HNE–*N*-acetylcysteine and HNE–glutathione adducts in a solvent [10% (vol/vol) methanol/chloroform]-extractable form. Treatment of proteins with HNE led to the disappearance of protein sulfhydryl groups. However, <10% of the labeled adducts obtained after subsequent reduction with NaB[^{3}H]H$_4$ could be released in a solvent-extractable form upon treatment with Raney nickel. This and the observation that HNE reacts with proteins lacking a sulfhydryl group attests to the fact that HNE can react with amino acid residues other than cysteinyl residues.**

Lipid peroxidation has been associated with important pathophysiological events in a variety of diseases, drug toxicities, and traumatic or ischemic injuries. Esterbauer and his colleagues (1–8) suggest that this toxicity is largely attributable to α,β-unsaturated aldehydes that are produced during lipid peroxidation. In particular, 4-hydroxynonenal (HNE) is a major product of lipid peroxidation and is believed to be mainly responsible for the observed cytopathological effects, which include: the inactivation of enzymes (1); lysis of erythrocytes (3); chemotactic activity of neutrophils (4); and the inhibition of protein and DNA synthesis (5). The cytotoxic effect of 4-hydroxy-2-alkenals is due, in part, to their facile reaction with sulfhydryl groups to form thioether adducts via a Michael addition mechanism (9–11). Because these protein thioethers contain a reactive aldehydic function, their derivatization with 2,4-dinitrophenylhydrazine has been used as a measure of protein-bound alkenals (1). However, protein carbonyl groups are also formed in the metal-catalyzed oxidation of side chains of amino acid residues (12–15), in the oxidative cleavage of proteins by α-amido transfer mechanisms (16), and in the glycation of proteins (17). Because the generation of protein carbonyl groups is associated with a number of pathological conditions (18–21), methods are needed to differentiate between various modes of carbonyl-group formation, to assess the contributions of a given mode to a particular disease state. To this end, we describe here a method for the detection and quantitation of carbonyl groups associated with the conjugation of protein sulfhydryl groups with lipid-peroxidation products. This method takes advantage of the fact that the lipid-derived carbonyl adducts can be reduced to stable radioactive derivatives by reduction with NaB[^{3}H]H$_4$ and that the labeled adduct can be released in an organic solvent extractable form by cleavage of the thioether linkage upon treatment with Raney nickel (22–25).

MATERIALS AND METHODS

Materials. A stock solution of *trans*-4-hydroxy-2-nonenal was prepared by the acid treatment (1 mM HCl) of HNE diethylacetal, which was provided by H. Esterbauer (University of Graz, Austria). Concentration of the HNE stock solution was determined from the molar extinction coefficient of HNE at 224 nm (9). Purity of the stock solution was also assessed by reverse-phase HPLC. ^{3}H-labeled sodium borotetrahydride (5–15 Ci/mmol; 1 Ci = 37 GBq) was obtained from DuPont/New England Nuclear. The Raney nickel-activated catalyst, L-cysteine, *N*-acetylcysteine, glutathione (GSH) (reduced form), bovine serum albumin, and *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase were obtained from Sigma. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was obtained from Calbiochem. *Escherichia coli* glutamine synthetase was prepared as described (26, 27). Raney nickel was rinsed thoroughly with water and ethanol before use.

HPLC. Reverse-phase HPLC was done on a Hewlett–Packard model 1090 chromatograph equipped with a Hewlett–Packard model 1040A diode-array UV detector. Formation of product was detected by HPLC, using an Apex octadecyl 5U column (0.46 × 15 cm). Separation was achieved by eluting with a linear gradient, in which the fraction of 0.05% trifluoroacetic acid solution was varied from 100 to 0% and the fraction of acetonitrile (vol/vol) was varied from 0 to 100% over a period of 0–20 min at a flow rate of 1 ml/min. The elution profiles were monitored by absorbance at 210 nm.

Reaction of *N*-Acetylcysteine and GSH with HNE. The HNE adducts of *N*-acetylcysteine and GSH were prepared by incubating 2 mM *N*-acetylcysteine or GSH with 2 mM HNE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, at 37°C for 2 hr. Formation of HNE–*N*-acetylcysteine and HNE–GSH adducts was determined by reverse-phase HPLC. The adducts were chemically characterized by mass spectrometric analyses with a californium-252 plasma desorption mass spectrometer. Quasimolecular ions, 342 $(M+Na)^+$, 345 $(M+Na+2H)^+$, and 364 $(M+2Na-H)^+$, were observed from the HNE–*N*-acetylcysteine adducts, and 486 $(M+Na)^+$ and 508 $(M+2Na-H)^+$ were observed from the HNE–GSH adduct.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HNE, 4-hydroxynonenal; GSH, glutathione.

*To whom reprint requests should be addressed at: National Institutes of Health, 9000 Rockville Pike, Building 3, Room 222, Bethesda, MD 20892.

Reduction of HNE Adducts of *N*-Acetylcysteine and GSH. After a 2-hr incubation, the reaction mixtures (400 μl) containing 2 mM of the HNE adduct of either *N*-acetylcysteine or GSH were incubated with 40 μl of 10 mM EDTA, 40 μl of 1 M NaOH, and 40 μl of 100 mM $NaBH_4$ in 0.1 M NaOH. After 1 hr at 37°C, the remaining borohydride was decomposed by adding 200 μl of 1 M HCl, and the samples reduced with $NaBH_4$ were concentrated by vacuum centrifugation (Savant Speed-Vac), resuspended in water, and samples were applied to reverse-phase HPLC. Reduced samples of HNE–*N*-acetylcysteine gave quasimolecular ions, 344 $(M+Na)^+$ and 366 $(M+2Na-H)^+$, and HNE–GSH gave 488 $(M+Na)^+$ and 510 $(M+2Na-H)^+$ in mass spectrometry.

Cleavage of ^{3}H-Labeled HNE Adducts of *N*-Acetylcysteine and GSH by Raney Nickel. After reduction with $NaB[^3H]H_4$ as described above, the samples were loaded on a Sep-Pak C-18 cartridge (Waters) and eluted with 5 ml of water and 5 ml of methanol. The methanol fraction was concentrated by vacuum centrifugation and resuspended in 200 μl of methanol; a sample was then applied to reverse-phase HPLC. One-milliliter fractions were collected, and the amount of ^{3}H incorporation in each fraction was monitored by scintillation counting. The fractions that contained the ^{3}H-labeled products were collected and evaporated under nitrogen.

The ^{3}H-labeled samples were solubilized in 500 μl of the guanidine hydrochloride solution, which contained 8 M guanidine hydrochloride, 13 mM EDTA, and 133 mM Tris, pH 7.2. Fifty-microliter aliquots of solubilized samples were added to reaction mixtures containing Raney nickel (400 mg) in 350 μl of the guanidine hydrochloride solution. After 15 hr at 55°C, the reaction mixtures were extracted twice with 500 μl of chloroform/methanol, 9:1, and dried with sodium sulfate. Then 50 μl of extract was added to 5 ml of Aquasol scintillation fluid (DuPont/New England Nuclear), and the radioactivity in this mixture was counted to measure the amount of released labeled product. Control samples received no catalyst.

One-hundred microliters of the chloroform/methanol extracts was also evaporated under nitrogen and resuspended in 100 μl of methanol; a sample was then applied to reverse-phase HPLC. One-milliliter fractions were collected, and 100-μl samples were subsequently used to determine the amount of radioactivity released in each fraction.

Reaction of Proteins with HNE Followed by ^{3}H-Labeling and Raney Nickel Treatment. One milligram of the protein substrate (either glyceraldehyde-3-phosphate dehydrogenase, glutamine synthetase, bovine serum albumin, or glucose-6-phosphate dehydrogenase) was incubated with 2 mM HNE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2. After 2 hr at 37°C, 400 μl of the reaction mixture was treated with 10% trichloroacetic acid (wt/vol, final concentration). The protein precipitate was collected by centrifugation (11,000 × *g*, 3 min) and was dissolved in 400 μl of the guanidine hydrochloride solution. The solutions were mixed with 0.1 M EDTA (40 μl) and 1 M NaOH (40 μl) in a 1.5-ml Sarstedt tube fitted with O ring and a cap. Forty microliters of 0.1 M $NaB[^3H]H_4$ in 0.1 M NaOH was added to each protein mixture. After incubation for 1 hr at 37°C, 100 μl of 1 M HCl was added, and then the mixture was applied to a PD-10 column (Sephadex G-25), equilibrated in 6 M guanidine hydrochloride, to separate the ^{3}H-labeled protein from radioactive contaminants. The amount of protein recovered was determined by using the molar extinction coefficients of 10.2 at 278 nm for glyceraldehyde-3-phosphate dehydrogenase, 3.87 at 290 nm for glutamine synthetase, 66 at 280 nm for bovine serum albumin, and 11.5 at 280 nm for glucose-6-phosphate dehydrogenase.

^{3}H-Labeled proteins were treated with Raney nickel as described in the previous section. The released products from the HNE-modified glyceraldehyde-3-phosphate dehydrogenase were analyzed by HPLC by using the same procedures as those from HNE-*N*-acetylcysteine and -GSH adducts.

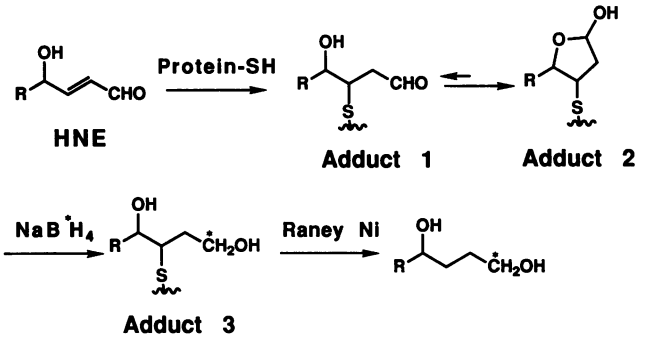

FIG. 1. Experimental scheme to determine HNE covalently attached to protein sulfhydryl groups by means of a thioether linkage. *, Tritium-labeled sites.

Reduction of HNE with $NaB[^3H]H_4$. HNE (2 mM) in 50 mM sodium phosphate buffer, pH 7.2, was treated with 40 μl of 100 mM $NaB[^3H]H_4$ for 1 hr at 37°C. The reaction was terminated by adding 100 μl of 1 M HCl. The products were analyzed by HPLC with the same procedures as those from HNE–*N*-acetylcysteine and –GSH adducts.

RESULTS

^{3}H-Labeling of HNE Adducts of *N*-Acetylcysteine and GSH. Our strategy for estimating the amount of thioether adducts formed by the reaction of HNE with protein sulfhydryl groups is illustrated in Fig. 1. The method is based on the fact that the thioether adducts possess a carbonyl group (Fig. 1, adducts 1 and 2), which upon reduction with $NaB[^3H]H_4$ is converted to the ^{3}H-labeled dihydroxy derivative (adduct 3). Desulfurization of adduct 3 by treatment with Raney nickel releases the labeled dihydroxy moiety, which after extraction into an organic solvent (10% methanol/90% $CHCl_3$) can be measured.

To test the validity of this procedure, the thioether adducts of *N*-acetylcysteine and GSH with HNE were prepared (9–11, 28). We confirmed that the reaction with HNE leads to complete loss of sulfhydryl groups. When these reaction mixtures were fractionated by HPLC, a number of products were detected (Figs. 2 and 3). Although these products have not been characterized definitely, they presumably represent thioether derivatives (Fig. 1, adducts 1 and 2), and for GSH, perhaps also the Schiff base derivative resulting from the interaction of the aldehyde moiety of HNE with the α-amino nitrogen of the glutamyl residue (see *Discussion*). After treatment of the reaction mixtures with $NaBH_4$, the elution profiles of the products markedly changed, due probably to

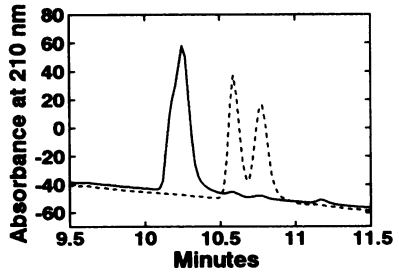

FIG. 2. HPLC profiles of HNE–*N*-acetylcysteine adducts before (- - -) and after (——) reduction with $NaBH_4$. *N*-acetylcysteine (2 mM) was incubated with 2 mM HNE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 2 hr at 37°C. A 100-μl sample of the mixture was then treated with 10 mM EDTA (10 μl) and 0.1 M NaOH (10 μl) for 1 hr at 37°C with or without 100 mM $NaBH_4$ (10 μl).

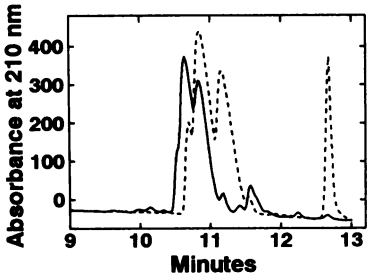

FIG. 3. HPLC profiles of HNE–GSH adducts before (- - -) and after (——) reduction with $NaBH_4$. GSH (2 mM) was incubated with 2 mM HNE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 2 hr at 37°C. A 100-μl sample of the mixture was then treated with 10 μl each of 10 mM EDTA and 0.1 M NaOH for 1 hr at 37°C with and without 10 μl of 100 mM $NaBH_4$.

their reduction to the corresponding alcohols and secondary amines. As noted in *Materials and Methods*, the mass spectrometric analyses of the *N*-acetylcysteine and GSH derivatives are consistent with the anticipated thioether structures. This conclusion is supported by the absence of the characteristic UV absorption spectrum of HNE, which would accompany addition of the sulfhydryl groups to the 2,3-double bond via a Michael-type mechanism.

Raney Nickel Treatment of HNE Adducts with *N*-Acetylcysteine and GSH. To determine whether the thioether linkage of HNE adducts of *N*-acetylcysteine and GSH could be cleaved by Raney nickel, the aldehyde group of their adducts were first reduced to their corresponding labeled hydroxy derivatives by treatment with $NaB[^3H]H_4$ as described in *Materials and Methods*. The labeled alcohols were then treated with Raney nickel. Fig. 4 shows that upon treatment with Raney nickel, >85% of the radiolabel was present in a form readily extractable into a chloroform/methanol (vol/vol), 9:1, solvent mixture. Before Raney nickel treatment, only 1 and 25% of the radioactivity in the *N*-acetylcysteine and GSH adducts, respectively, could be extracted by the solvent. Therefore, the Raney nickel treatment obviously resulted in cleavage of the thioether bond. The small amount (25%) of solvent-extractable radioactivity present in the HNE–GSH derivative before Raney nickel treatment has not been positively identified. However, the reduced forms of free HNE (i.e., 1,4-dihydroxynonane) and its GSH adduct coelute in the chromatographic system used for isolating the GSH adduct. Therefore, the solvent-extractable radioactivity present in the GSH adduct before Raney nickel treatment could reflect

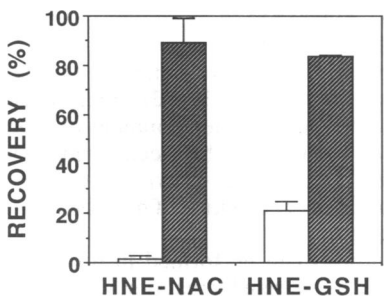

FIG. 4. Recovery of radioactivity from ^3H-labeled HNE adducts of *N*-acetylcysteine (NAC) and GSH after treatment with Raney nickel. HNE adducts of *N*-acetylcysteine and GSH were treated with $NaB[^3H]H_4$ and then incubated in 8 M guanidine hydrochloride/133 mM Tris/13 mM EDTA solution for 20 hr at 55°C with (shaded bars) and without Raney nickel (open bars). Reaction mixtures were extracted twice with 500 μl of chloroform/methanol, 9:1, and dried with sodium sulfate; then 50 μl of extracts was added to 5 ml of Aquasol scintillation fluid to measure the amount of released radioactivity.

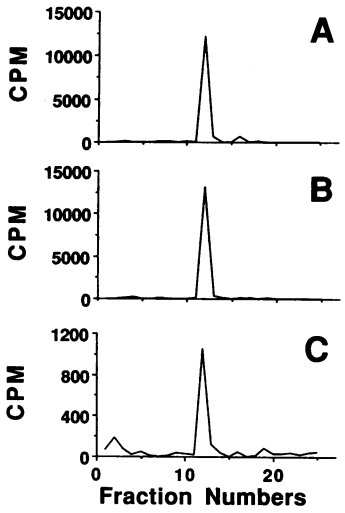

FIG. 5. HPLC fractionation of released products from ^3H-labeled products of HNE–*N*-acetylcysteine (*A*), HNE–GSH (*B*) adducts, and glyceraldehyde-3-phosphate dehydrogenase modified with HNE (*C*).

contamination of the adduct preparation with radiolabeled 1,4-dihydroxynonane or other reduced forms of HNE. When the chloroform/methanol extracts of the Raney nickel-treated *N*-acetylcysteine and GSH adducts were fractionated by the HPLC technique, samples contained a single major radioactive component—probably 1,4-dihydroxynonane, with a retention time of 11.5 min (Fig. 5 *A* and *B*). A reference sample prepared by the reduction of HNE with $NaB[^3H]H_4$ has the same retention time, thus providing support for the assumption that the Raney nickel cleavage product is 1,4-dihydroxynonane. Thus, it is evident that the above procedure, involving the ^3H-labeling of the thioether adducts followed by solvent extraction of the radioactive Raney nickel cleavage product, can determine the fraction of the total carbonyl content of a protein due to the conjugation of lipid-derived α,β-unsaturated aldehydes with protein thiol groups.

^3H-Labeling of HNE-Modified Proteins Followed by Raney Nickel Treatment. Four proteins that vary with respect to number and reactivity of sulfhydryl groups per subunit were tested for their ability to form Raney nickel-cleavable conjugates with HNE. Table 1 shows that glyceraldehyde-3-phosphate dehydrogenase contains four sulfhydryl groups per subunit; however, only 3.2 of these sulfhydryl groups were lost during incubation with HNE. Moreover, the total amount of HNE (5.3 mol/mol of subunit) bound to the protein was significantly greater than the amount of sulfhydryl groups that disappeared. Evidently a substantial amount of

Table 1. ^3H incorporation into HNE-modified proteins and recovery of radioactivity after treatment with Raney nickel

Protein	Cysteine per subunit	^3H incorporation, mol/mol of subunit	^3H recovery, mol/mol of subunit	Yield, %
G-3-PDH	4	5.3	0.54	10.2
G-6-PDH	0	1.5	0.03	1.8
GlnS	4	2.3	0.17	7.4
BSA	1	6.6	0.19	2.8

One milligram of each protein was incubated with 2 mM HNE in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 2 hr at 37°C. Both ^3H incorporation and recovery of radioactivity were determined according to the procedure outlined in text. G-3-PDH, glyceraldehyde-3-phosphate dehydrogenase; G-6-PDH, glucose-6-phosphate dehydrogenase; GlnS, glutamine synthetase; BSA, bovine serum albumin.

the HNE reacted with amino acid residues other than cysteine residues. Furthermore, after reduction with NaB[^{3}H]H$_4$, only 0.54 mol/mol of the labeled adduct was released in a solvent-extractable form upon incubation with Raney nickel. Thus, only 17% of the modified cysteine residues was present as the simple HNE–thioether adducts.

That HNE reacts with groups other than sulfhydryl group on proteins is evident also from the fact that a protein that contains no cysteine residue (i.e., glucose-6-phosphate dehydrogenase from *L. mesenteroides*), nevertheless, bound 1.5 mol of HNE per mol of subunit. As expected, an insignificant amount (1.8%) of the HNE bound to this protein could be released by treatment with Raney nickel.

Although *E. coli* glutamine synthetase contains four cysteine residues per subunit, only 2.3 equivalents of HNE were bound per subunit, and <10% of that bound could be released upon treatment with Raney nickel. Failure to react with all four of the sulfhydryl groups of glutamine synthetase is likely due to the fact that in the native configuration, the SH groups are inaccessible to reaction with sulfhydryl reagents (29).

That HNE reacts with amino acid residues other than cysteine residues is most clearly demonstrated by the experiment with bovine serum albumin. This protein contains only one free sulfhydryl group, but it bound 6.6 mol of HNE per mol.

Although only a small fraction of the ^{3}H-labeled HNE–protein adducts were converted to solvent-extractable material by the Raney nickel treatment, the extractable material from the HNE-modified glyceraldehyde-3-phosphate dehydrogenase yielded a single radioactive peak upon HPLC analysis (Fig. 5*C*). Elution time of this material was identical to that obtained from the ^{3}H-labeled HNE–*N*-acetylcysteine and HNE–GSH adducts (Fig. 5 *A* and *B*) and of the synthetic product tentatively identified as 1,4-dihydroxynonane.

DISCUSSION

4-Hydroxyalkenals are known as diffusible cytotoxic products that originate from the peroxidative breakdown of polyunsaturated fatty acids containing more than one methylene-interrupted double bond, such as linoleic acid and arachidonic acid (2). HNE, as well as other α,β-unsaturated aldehydes, are known electrophilic agents that react rapidly with the sulfhydryl moiety in cysteine, GSH, and protein. Esterbauer *et al.* (11) demonstrated that 4-hydroxy-2-alkenals are more reactive with GSH than are α,β-unsaturated aldehydes lacking the C-4 hydroxyl group (namely, crotonaldehyde). The electron-withdrawing 4-hydroxy group has been postulated to make C-3 of the 4-hydroxyalkenals more electropositive and, thus, more susceptible to nucleophilic attack. The reaction of HNE with a mercaptan is expected to yield a saturated aldehyde as a primary product in which the sulfhydryl group of the mercaptan is attached in thioether linkage to the C-3 of the HNE (Fig. 1). Subsequently, the free aldehyde moiety of the primary product may react with the 4-hydroxyl group to form a cyclic hemiacetal (lactol ring) derivative (1, 2, 9–11). It has been suggested that the oxo–cyclo equilibrium would favor hemiacetal formation (10).

In the present study, the reaction of HNE with *N*-acetylcysteine and GSH gave rise to multiple products, as detected by HPLC analysis (Figs. 2 and 3). Because only limited amounts of HNE were available for these studies, it was not possible to rigorously characterize the various products. It is significant, however, that, after treatment first with NaB[^{3}H]H$_4$ and then with Raney nickel, only one major solvent-extractable product was obtained, which accounted for 85% or more of the total radioactive material formed. It is, therefore, possible that the multiplicity of primary products formed represent closely related forms (isomers) of the HNE–*N*-acetylcysteine and –GSH adducts, which upon cleavage with Raney nickel yield a common product—i.e., probably 1,4-dihydroxynonane. The HNE used in this study is a racemic mixture of (4*R*)- and (4*S*)-isomers. The hemiacetal derivatives would, therefore, contain chiral centers at C-1, C-3, and C-4 of the nonenal moiety. Accordingly, the hemiacetal derivatives of *N*-acetylcysteine and GSH could be composed of eight isomers and, after reduction with NaBH$_4$, should yield two pairs of diastereomers. In addition, reaction of the aldehyde moiety of HNE with the α-amino group of glutamyl moiety of GSH could lead to the formation of Schiff base derivatives. Whereas the precise identities of the several products formed remain to be determined, it is evident from the data presented here that the reduction of protein carbonyl groups with NaB[^{3}H]H$_4$ and subsequent measurement of the organic solvent-extractable radioactive products, which are released by Raney nickel treatment, provide a means of determining the fraction of total free carbonyl groups introduced into proteins via the reaction of α,β-unsaturated aldehydic lipid-peroxidation products with protein sulfhydryl groups. By this criterion, aldehydic thioether adducts were demonstrated to account for <10% of the total protein carbonyl groups associated with the modified proteins that accumulate in liver hepatocytes of old rats (unpublished observation).

This percentage could, however, underestimate the amount of protein damage that occurs from reactions with lipid-peroxidation products. As noted here (Table 1), HNE reacts with the side chains of amino acid residues other than cysteine to form products that are converted to radioactive derivatives after treatment with NaB[^{3}H]H$_4$. These include reactions with lysine amino groups of proteins to form Schiff bases and with histidine residues of proteins (30). Why some products formed by the interaction of HNE with protein sulfhydryl groups are not converted to solvent-extractable derivatives by the Raney nickel treatment remains to be determined. When glyceraldehyde-3-phosphate dehydrogenase was treated with HNE, 3.2 sulfhydryl groups per subunit were modified, but after NaBH$_4$ reduction, only 0.54 equivalent of solvent-extractable radiolabeled product was formed. Among other possibilities, such behavior could be explained if the reaction of HNE with the sulfhydryl group to form a thioether derivative is followed by a secondary reaction in which the aldehyde group of the thioether reacts with the amino group of a proximal lysine residue of the protein to form a Schiff base. Upon NaBH$_4$ treatment, the Schiff base would be stabilized by its conversion to a stable secondary amine. Then, upon Raney nickel treatment, cleavage of the thioether linkage would occur, but the HNE adduct would remain tethered to the protein by means of its attachment to the lysine residue.

Immunochemical data have indicated that HNE-modified proteins are formed *in vivo* (31, 32). Because HNE-modified proteins are antigenic, the development of monoclonal antibodies that can discriminate between various kinds of HNE–amino acid derivatives may provide a way to evaluate the relative importance of each kind of modification to the overall protein damage.

Carbonyl groups can be formed in proteins by nonenzymatic reactions other than by reaction with lipid-peroxidation products to form thioether adducts (12–17, 30). As already noted, the development of methods that can discriminate between various pathways of protein carbonyl-group generation is critical to assessing the role of a given pathway in a particular pathological condition. The method described here allows quantitation of carbonyl groups arising from the conjugation of lipid-derived α,β-unsaturated aldehydes with protein sulfhydryl groups.

We thank Dr. Hermann Esterbauer (University of Graz, Austria)

for the gift of HNE. We also thank Dr. Rodney L. Levine for many helpful discussions, Dr. Edward Sokoloski of the Laboratory of Chemistry, National Heart, Lung, and Blood Institute, for obtaining the mass spectra, and Barbara Berlett for assistance in some of the analytical procedures.

1. Schauenstein, E. & Esterbauer, H. (1979) *Ciba Found. Symp.* **67**, 225–244.
2. Esterbauer, H., Schaur, R. J. & Zollner, H. (1991) *Free Radical Biol. Med.* **11**, 81–128.
3. Benedetti, A., Comporti, M. & Esterbauer, H. (1980) *Biochim. Biophys. Acta* **620**, 281–296.
4. Cuzio, M., Esterbauer, H., Mauro, C. D., Cecchini, G. & Dianzani, M. U. (1986) *Biol. Chem. Hoppe-Seyler* **367**, 321–329.
5. Esterbauer, H., Zollner, H. & Lang, J. (1985) *Biochem. J.* **228**, 363–373.
6. Benedetti, A., Esterbauer, H., Ferrali, M., Fulceri, R. & Comporti, M. (1982) *Biochim. Biophys. Acta* **711**, 345–356.
7. Esterbauer, H., Cheeseman, K. H., Dianzini, M. U., Poli, G. & Slater, T. F. (1982) *Biochem. J.* **208**, 129–140.
8. Benedetti, A., Pompella, A., Fulceri, R., Romani, A. & Comporti, M. (1986) *Biochim. Biophys. Acta* **876**, 658–666.
9. Schauenstein, E., Taufer, M., Esterbauer, H., Kylianek, A. & Seelich, T. (1971) *Monatsh. Chem.* **102**, 517–529.
10. Esterbauer, H., Zollner, H. & Scholz, N. (1975) *Z. Naturforsch. C Biosci.* **30**, 466–473.
11. Esterbauer, H., Ertl, A. & Scholz, N. (1976) *Tetrahedron* **32**, 285–289.
12. Amici, A., Levine, R. L., Tsai, L. & Stadtman, E. R. (1989) *J. Biol. Chem.* **264**, 3341–3346.
13. Stadtman, E. R. (1990) *Free Radical Biol. Med.* **9**, 315–325.
14. Stadtman, E. R. & Oliver, C. N. (1991) *J. Biol. Chem.* **266**, 2005–2008.
15. Stadtman, E. R. (1991) *Biochemistry* **29**, 6323–6331.
16. Bateman, R. C., Jr., Youngblood, W. W., Busby, W. H. & Kizer, J. S. (1985) *J. Biol. Chem.* **260**, 9088–9091.
17. Dunn, J. A., Ahmed, M. U., Murtiashaw, M. H., Richardson, J. M., Walla, M. D., Thorpe, S. R. & Baynes, J. W. (1990) *Biochemistry* **29**, 10964–10970.
18. Starke-Reed, P. E. & Oliver, C. N. (1989) *Arch. Biochem. Biophys.* **275**, 559–567.
19. Oliver, C. N., Starke-Reed, P. E., Stadtman, E. R., Liu, G. J., Carney, J. M. & Floyd, R. A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5144–5147.
20. Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Landum, R. W., Cheng, M. S., Wu, J. F. & Floyd, R. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 3633–3636.
21. Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A. & Markesbery, W. R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10540–10543.
22. Scaffer, M. H. & Stark, G. R. (1976) *Biochem. Biophys. Res. Commun.* **71**, 1040–1047.
23. Farnsworth, C. C., Wolda, L. S., Gelb, M. H. & Glomset, J. A. (1989) *J. Biol. Chem.* **264**, 20422–20429.
24. Rilling, M. C., Breunger, E., Epstein, W. W. & Crain, P. F. (1990) *Science* **247**, 318–320.
25. Farnsworth, C. C., Gelb, M. H. & Glomset, J. A. (1990) *Science* **247**, 320–322.
26. Miller, R. E., Shelton, E. & Stadtman, E. R. (1974) *Arch. Biochem. Biophys.* **163**, 155–171.
27. Roseman, J. E. & Levine, R. L. (1987) *J. Biol. Chem.* **262**, 2101–2110.
28. Ishikawa, T., Esterbauer, H. & Sies, H. (1986) *J. Biol. Chem.* **261**, 1576–1581.
29. Shapiro, B. M. & Stadtman, E. R. (1967) *J. Biol. Chem.* **242**, 5069–5079.
30. Uchida, K. & Stadtman, E. R. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4544–4548.
31. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. & Wiztum, J. L. (1989) *N. Engl. J. Med.* **320**, 915–924.
32. Palinski, W., Rosenfeld, M. E., Yla-Herttuala, S., Gurtner, G. C., Socher, S. S., Butler, S. W., Parthasarathy, S., Carew, T. E. & Steinberg, D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1372–1376.