

Modification of histidine residues in proteins by reaction with 4-hydroxynonenal

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ABSTRACT We find that histidine residues in proteins are major targets for reaction with the lipid peroxidation product 4-hydroxynonenal (HNE). Reaction of insulin (which contains no sulfhydryl groups) with HNE leads to the generation of HNE-protein adducts, which are converted to radioactive derivatives upon subsequent treatment with $\text{NaB}[\text{}^3\text{H}]\text{H}_4$. Amino acid analysis of the modified protein showed that the HNE treatment leads to the selective loss of histidine residues and the stoichiometric formation of ^3H -labeled amino acid derivatives. The same labeled products were detected in acid hydrolysates of polyhistidine and *N*-acetylhistidine after their reactions with HNE and $\text{NaB}[\text{}^3\text{H}]\text{H}_4$. The reaction of *N*-acetylhistidine with HNE led to the production of two compounds. Upon acid hydrolysis, both derivatives yielded stoichiometric amounts of histidine. However, after reduction with NaBH_4 , acid hydrolysis led to a mixture of amino acid derivatives [presumably, isomeric forms of *N* $^{\pi}$ (*N'*)-1,4-dihydroxynonan-ylhistidine] that were indistinguishable from those obtained from insulin and polyhistidine after similar treatment. Although other possibilities are not excluded, it is suggested that the modification of histidine residues in proteins by HNE involves a Michael-type addition of the imidazole nitrogen atom of histidine to the α,β -unsaturated bond of HNE, followed by secondary reaction involving the aldehyde group with the C-4 hydroxyl group of HNE. The reaction of histidine residues with HNE provides the basis for methods by which the contributions of HNE in the modification of proteins can be determined.

4-Hydroxynonenal (HNE) is one of the major products of membrane peroxidation and has been shown to have a number of adverse biological effects such as high toxicity to mammalian cells (1, 2), the lysis of erythrocytes (1), inactivation of enzymes (3), and inhibition of the synthesis of DNA and protein (3, 4). These effects have been attributed in part to the fact that HNE reacts readily with sulfhydryl groups of proteins and low molecular weight metabolites, such as cysteine and glutathione, to yield stable thioether derivatives (5–7). Results of immunochemical studies with HNE-specific antibodies have shown that HNE-modified proteins are generated *in vivo* and, therefore, may play a physiological role (8–11). As noted by Esterbauer *et al.* (12), elucidation of the chemistry of the reactions of HNE with proteins is fundamental to an understanding of the mechanism of HNE cytotoxicity and also to the characterization of the epitopes in HNE-modified proteins that are recognized by antibodies.

In the present paper, we demonstrate that, in addition to sulfhydryl groups and primary amino groups, the histidine residues of proteins are important targets for modification by HNE. In studies with insulin, which contains no sulfhydryl groups, it was shown that histidine is the only amino acid that is modified by exposure to HNE; the one lysine that is present in insulin was not modified under the same conditions. In addition, the results of model studies with *N*-acetyl-

histidine suggest that HNE and 2-nonenal react with the imidazole nitrogen atoms of histidine residues in proteins by a Michael-type addition reaction.

MATERIALS AND METHODS

Materials. The stock solution of *trans*-4-hydroxy-2-nonenal was prepared by the acid treatment (1 mM HCl) of HNE diethylacetal, which was generously provided by Hermann Esterbauer (University of Graz, Austria). Sodium borotetratritide (5–15 Ci/mmol; 1 Ci = 37 GBq) was obtained from DuPont/New England Nuclear, and poly(L-histidine), insulin, *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase, and bovine serum albumin were from Sigma. *N*- α -Acetyl-L-histidine and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase were obtained from Calbiochem; *trans*-2-nonenal was from Aldrich.

Instrumentation. Reverse-phase HPLC was performed on a Hewlett-Packard model 1090 chromatograph equipped with a Hewlett-Packard model 1040A diode array UV detector. Fast atom bombardment-mass spectrometry was performed with a JEOL JMS-SX102 mass spectrometer.

Incubation of Protein with HNE. The reaction mixture containing 1 mg of protein was incubated with 2 mM HNE in 1 ml of 50 mM sodium phosphate (pH 7.2) for 2 h at 37°C. After incubation, samples were assayed for carbonyl content (13), amino acid composition (14), and the ability to form ^3H -labeled derivatives upon treatment with $\text{NaB}[\text{}^3\text{H}]\text{H}_4$.

Reduction with NaBH_4 . A sample (100 μl) of the reaction mixture was treated with 10% (wt/vol) trichloroacetic acid (final concentration). The protein precipitate was dissolved with 400 μl of 8 M guanidine hydrochloride/13 mM EDTA/133 mM Tris-HCl, pH 7.2. The solution was mixed with 10 mM EDTA (40 μl) and 1 M NaOH (40 μl) in a 1.5-ml Sarstedt tube fitted with an O-ring and cap. Then, 40 μl of 0.1 M $\text{NaB}[\text{}^3\text{H}]\text{H}_4$ in 0.1 M NaOH was added and the mixture was incubated for 1 h at 37°C. After incubation, the reaction was terminated by the addition of 100 μl of 1 M HCl and the mixture was applied to a PD-10 column (Sephadex G-25), equilibrated in 6 M guanidine hydrochloride, to separate protein-bound radioactivity from free radioactivity. Recovery of protein was determined by protein assay reagent (Bio-Rad).

Amino Acid Analysis. A sample (100 μl) of the reaction mixture was treated with 10 mM EDTA (40 μl)/1 M NaOH (40 μl)/100 mM NaBH_4 or $\text{NaB}[\text{}^3\text{H}]\text{H}_4$ (40 μl) for 1 h at 37°C. The reduced protein was precipitated with 10% trichloroacetic acid and then hydrolyzed with 6 M HCl (200 μl) for 20 h at 110°C under nitrogen atmosphere. The hydrolyzed sample was evaporated to dryness and dissolved in 1 ml of 50 mM sodium phosphate (pH 8.0) containing 1 mM EDTA. The

Abbreviations: HNE, 4-hydroxynonenal; LDL, low density lipoprotein; 2,4-DNP, 2,4-dinitrophenylhydrazine; OPA, *o*-phthalaldehyde. *To whom reprint requests should be addressed at: National Institutes of Health, 9000 Rockville Pike, Building 3, Room 222, Bethesda, MD 20892.

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solution was diluted and 10 μ l was labeled with *o*-phthalaldehyde (OPA) for determination of amino acid composition by reverse-phase HPLC (15).

Sequencing Analysis of the HNE-Modified Insulin B Chain. A reaction mixture (1.0 ml) containing 1 mg of insulin, 2 mM HNE, and 50 mM sodium phosphate (pH 7.2) was incubated at 37°C. Then 20 μ l of 1 M NaOH and 20 μ l of dithiothreitol (10 mg/ml) were added, and after incubation for 10 min at room temperature, the mixture was treated with 20 μ l of iodoacetate (25 mg/ml) for 20 min at room temperature and then applied to an HPLC column. Separation of the A and B chains of insulin was achieved using a linear gradient in which the fraction of 0.05% trifluoroacetic acid was varied from 80 to 0% and the concentration of acetonitrile was varied from 20 to 80% (vol/vol) over 0–20 min at a flow rate of 1 ml/min. The elution profile was monitored at 210 nm. The fraction containing the modified histidine (the B chain) eluted at 9–10 min and was collected, and 30 μ g of the purified product was used for amino acid sequence determination.

Reaction of *N*-Acetylhistidine with HNE and 2-Nonenal. Reaction mixtures (1.0 ml) containing 50 mM sodium phosphate (pH 7.2) and either 2 mM HNE or 2 mM 2-nonenal were incubated at 37°C for 20 h. Formation of product was detected by HPLC, using an Apex Octadecyl 5U column (0.46 \times 15 cm). Separation of products 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 was varied from 0 to 100% over 0–20 min, at a flow rate of 1 ml/min. The elution profiles were monitored by absorbance at 210 nm. The *N*-acetylhistidine-HNE adducts were eluted between 11 and 14 min. The product mixture was collected and analyzed by fast atom bombardment-mass spectrometry.

RESULTS

4-HNE Modification of Insulin. In preliminary studies with several proteins, it became evident that, in addition to cysteine and lysine residues, the reaction with HNE leads to a significant loss of histidine residues. To facilitate characterization of the HNE-histidine adduct, insulin was selected for further studies because it contains no free sulfhydryl groups and contains only two histidine and one lysine residues per polypeptide chain. The HNE-modified insulin was prepared by incubating insulin (1 mg/ml) with 2 mM HNE for 2 h at 37°C. Upon treatment of the HNE-modified insulin with $\text{NaB}[\text{H}]_4$, 1.2 mol of ^3H were bound per mol of insulin added (Fig. 1). The ^3H -labeled protein was then subjected to acid hydrolysis and after derivatization with OPA, the amino acid composition of the mixture was determined by HPLC. The analysis showed that HNE modification is associated with the loss of 1.1 histidine residues per mol of insulin, and

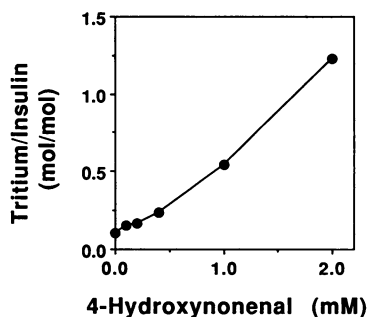


FIG. 1. ^3H incorporation into insulin treated with HNE. Insulin (1 mg/ml) was incubated with 0–2 mM HNE in 1 ml of 50 mM sodium phosphate (pH 7.2) for 2 h at 37°C. After treatment with $\text{NaB}[\text{H}]_4$, the amount of radioactivity incorporated into the protein was determined.

the formation of several ^3H -labeled amino acids whose OPA derivatives (peaks A–D) were eluted between leucine and lysine in the HPLC system used (Fig. 2). The same amino acids were detected in acid hydrolysates of poly(L-histidine) that had been treated with HNE and subsequently reduced with NaBH_4 (data not shown).

To determine which histidine residue of insulin is the target for HNE, A and B chains of modified insulin were resolved by treatment with dithiothreitol and then with iodoacetate under alkaline conditions (16). The B chain that contains the modified histidine was purified by HPLC and its amino acid sequence was determined by automated Edman degradation. The result demonstrated the loss of 73% of His⁵ and 69% of His¹⁰. It was thus obvious that HNE had reacted with both histidine moieties equally.

Whereas α,β -unsaturated aldehydes are believed to react readily with amino groups of proteins to form Schiff bases (11, 12, 17, 18), the single lysine residue of insulin was only slightly modified (0.03 mol/mol of protein) by the treatment with HNE.

4-HNE Adducts of *N*-Acetylhistidine. In an effort to determine the structure of HNE-modified histidine generated in insulin, the product formed by reaction of HNE with *N*-acetylhistidine was examined. When 50 mM *N*-acetylhistidine was incubated with 2 mM HNE for 20 h at 37°C, two products (H-1 and H-2) were detected (Fig. 3).

Borohydride reduction followed by acid hydrolysis and then OPA derivatization of the H-1 and H-2 product mixture gave the same peaks in amino acid analysis as those detected in the HNE-modified insulin. This confirms that the same adducts were generated in insulin exposed to HNE. In addition, when tested separately, amino acid analysis of H-1 generated peaks A and B, and analysis of H-2 generated peaks C and D. Among other possibilities, these results suggest that there are at least four isomeric forms of the HNE-histidine adduct.

The following lines of evidence suggest that the modification of *N*-acetylhistidine involves a Michael-type addition of the imidazole nitrogen atom to C-3 of HNE: (i) The UV light absorption spectrum of the *N*-acetylhistidine-HNE adducts has a maximum at 217 nm, whereas the spectrum of free HNE exhibits a maximum at 224 nm. This shift to a lower wavelength is characteristic of reactions involving the saturation of an aliphatic double bond. (ii) Positive-ion fast atom bom-

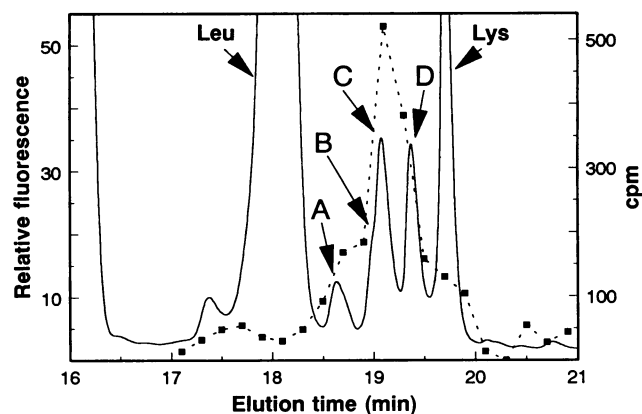


FIG. 2. Amino acid analysis of the HNE-modified insulin. Insulin (1 mg/ml) was incubated with 2 mM HNE in 1 ml of 50 mM sodium phosphate (pH 7.2) for 2 h at 37°C. After incubation, a sample of the mixture was treated with $\text{NaB}[\text{H}]_4$ and then hydrolyzed with 6 M HCl for 20 h at 110°C. The OPA derivatives of the amino acids were analyzed (fluorescence intensity) by HPLC (15). Simultaneously, fractions were collected every 12 sec and the radioactivity (cpm) was determined. Peaks A–D (solid line) represent the HNE-modified amino acids.

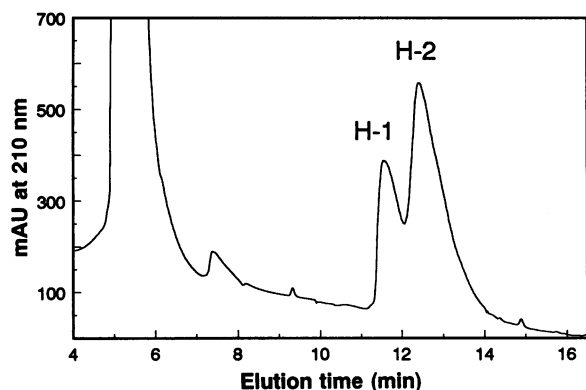


FIG. 3. HPLC profile of the reaction mixture of *N*-acetylhistidine exposed to HNE. *N*-Acetylhistidine (50 mM) in 1 ml of 50 mM sodium phosphate (pH 7.2) was incubated with 2 mM HNE for 20 h at 37°C. H-1 and H-2 represent HNE-modified *N*-acetylhistidine. A peak eluted between 4.5 and 7 min represents *N*-acetylhistidine. mAU, arbitrary units ($\times 10^{-3}$).

bardment-mass spectrometry analysis of the products gave the $M + H$ ion m/z 354, which would be expected from a Michael-type addition product. (iii) The reaction of HNE with *N*-acetylhistidine is associated with the loss of a histidine residue and the stoichiometric formation of a product that, upon reduction with $\text{NaB}[\text{H}]_4$, leads to incorporation of one equivalent of ^3H into the product. Nevertheless, the Michael addition mechanism appears inconsistent with the fact that most of the *N*-acetylhistidine-HNE adduct does not react with 2,4-dinitrophenylhydrazine (2,4-DNP) to form a hydrazone (data not shown). Michael addition reactions should not affect the aldehyde function of HNE, unless the aldehyde moiety of the primary product reacts with the hydroxyl group at the C-4 position to form a hemiacetal (Fig. 4). Even so, the hemiacetal derivative should react with 2,4-DNP to form a hydrazone (5).

Substitution of 2-Nonenal for HNE. To eliminate the possibility of hemiacetal formation, we studied the reaction of 2-nonenal with *N*-acetylhistidine. In contrast to the results obtained with HNE, the principal product formed with 2-nonenal did react with 2,4-DNP, as expected for a Michael addition mechanism. This result indicates also that the failure to detect a carbonyl function in the HNE product reflects an interaction between the aldehyde and the C-4 hydroxyl group (hemiacetal formation?) of the HNE derivative. Esterbauer (5) reported that the aldehyde moiety of thioethers produced by the reaction of HNE with the sulfhydryl group of mercaptans exist almost exclusively as the hemiacetal and that the hemiacetal does react with 2,4-DNP (3, 7). We confirmed that *N*-acetylcysteine reacts with HNE to form a thioether, but under our conditions, most of the thioether did not react with 2,4-DNP. Therefore, the question of whether hemiacetals are formed, and if so, whether they will react with 2,4-DNP remains unsettled.

It is noteworthy that treatment of the *N*-acetylhistidine-HNE adduct with NaBH_4 stabilizes the histidyl-HNE linkage. If the reduction step is omitted, acid hydrolysis of the

Table 1. Loss of histidine and concomitant formation of HNE-histidine adducts

Protein	Histidine, mol/mol of subunit		HNE-histidine formed, mol/mol of subunit
	Before	After	
Insulin	2.12	1.02	1.03
Glyceraldehyde-3-phosphate dehydrogenase	9.46	4.58	4.30
Glucose-6-phosphate dehydrogenase	5.84	4.57	0.26
Bovine serum albumin	16.60	9.39	6.11

Each protein (1 mg) was incubated with 2 mM HNE in 1 ml of 50 mM sodium phosphate (pH 7.2) for 2 h at 37°C. Histidine and HNE-histidine adducts before and after incubation were determined.

N-acetylhistidine-HNE adduct leads to quantitative release of the histidyl moiety as free histidine. These observations are consistent with the Michael addition mechanism, provided that reversal of the addition reaction is catalyzed by strong acid. If so, reduction of the aldehyde moiety would preclude reversibility and, thereby, lead to formation of an acid-stable derivative.

Formation of HNE-Histidine Adducts in Proteins. In the HPLC system used, the HNE-histidine adducts can be clearly separated from all other normal amino acids. Therefore, the procedure used here can be used to quantitate the amount of HNE-modified histidine residues in proteins. It is generally accepted that lysine and cysteine residues of proteins are primary targets for reaction with HNE. The data in Table 1 show that histidine residues are also major targets. About one-half of the histidine residues in insulin, glyceraldehyde-3-phosphate dehydrogenase, and bovine serum albumin were modified by HNE under our experimental conditions. In contrast, the histidine residues in glucose-6-phosphate dehydrogenase from *L. mesenteroides* were not modified by HNE, whereas treatment of this enzyme with HNE did result in a loss of lysine residues (data not shown).

DISCUSSION

The cytotoxic effects of 4-hydroxy-2-alkenals have been attributed to their facile reaction with sulfhydryl groups to form stable thioether adducts by a Michael addition mechanism and/or reaction of the aldehyde moiety with the ϵ - NH_2 groups of lysyl residues to form Schiff bases (5-7). We show here that HNE reacts also with histidine residues in proteins. It is therefore likely that the modification of histidine residues of proteins also contributes to the cytotoxic action of 4-hydroxy-2-alkenals.

The proposition that HNE reacts with the imidazole nitrogen of histidine, by a Michael addition mechanism, is not without precedence. α,β -Unsaturated sugar aldehydes have been shown to react with purines by a Michael-type mechanism to yield isonucleosides (19).

It is generally believed that HNE reacts with the amino groups of lysine residues of proteins to form Schiff base

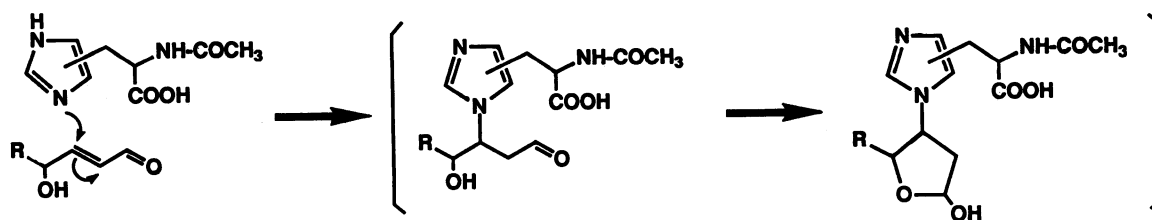


FIG. 4. Proposed mechanism for formation of HNE-histidine adducts.

conjugates (11, 12, 17, 18). Treatment of such conjugates with $\text{NaB}^3\text{H}]\text{H}_4$ would lead to the production of radiolabeled secondary amines. Therefore, to the extent that Schiff base adducts are formed, they would introduce error into the $\text{NaB}^3\text{H}]\text{H}_4$ procedure for the measurement of protein carbonyl groups. However, the presence of HNE-protein Schiff base conjugates will not interfere with the measurement of protein carbonyl groups by the 2,4-DNP procedure. Reaction of such conjugates with 2,4-DNP would involve cleavage of the HNE-Schiff base adduct and the formation of the free HNE-hydrazone, which would be separated from the unmodified protein by the procedure used. It is therefore significant that results of preliminary experiments using glucose-6-phosphate dehydrogenase and poly(L-lysine) have shown that conjugates formed by the interaction of HNE with amino groups of lysyl side chains of those polypeptides are not cleaved by 2,4-DNP to yield free HNE hydrazones but react with 2,4-DNP to form bona fide protein hydrazone derivatives (unpublished data). This suggests that HNE may undergo a Michael-type addition reaction with the amino groups of lysine side chains to form stable secondary amines, which can further react with either $\text{NaB}^3\text{H}]\text{H}_4$ or 2,4-DNP to yield radiolabeled protein or protein hydrazone derivatives, respectively.

Benedetti *et al.* (20) have suggested that the carbonyl groups of microsomal proteins reflect the conjugation of lipid peroxidation products with protein sulfhydryl groups. In light of the results presented here and results of studies on the modification of protein sulfhydryl groups with HNE (21), it is evident that measurement of lipid-derived protein carbonyl groups by the borotritide procedure could be misleading. However, validity of the borotritide technique is not compromised by adducts formed between histidine and 2-alkenals that do not contain a hydroxyl group at the C-4 position, since as noted here the adduct formed with 2-nonenal does react with 2,4-DNP.

It is noteworthy that in addition to the reactions of lipid peroxidation products with cysteine, histidine, and lysine residues of proteins, protein carbonyl groups can arise from the oxidation of amino acid side chains by both metal ion-dependent (22–24) and -independent (25) oxygen-free-radical-mediated mechanisms, by oxidative cleavage of peptide bonds by the α -amido transfer mechanism (26), and by glycation of protein amino groups (27). The accumulation of catalytically inactive or less active forms of enzymes during aging and in some pathological states is associated with an increase in the level of highly reactive carbonyl groups (28–31). It is therefore of considerable interest to develop analytical procedures that can be used to differentiate between the various mechanisms of generating carbonyl groups, to assess the contribution of each mechanism to the observed age-related changes and in various pathological conditions. The results described here provide the basis of a method that can be used to estimate the amount of protein carbonyl groups that is due to conjugation of HNE with histidine residues.

As shown in Fig. 3, the reaction of HNE with *N*-acetyl-histidine generated major isomers (H-1 and H-2), which we assume represent N^{π} - and N^{τ} -substituted adducts of the imidazole ring. Acid hydrolysis followed by amino acid analysis of their OPA derivatives demonstrated that both H-1 and H-2 were also composed of isomers (peaks A–D). We have not carried out a rigorous characterization of these isomers; however, the multiplicity of primary products formed may represent closely related forms (isomers) of the HNE-histidine adducts. This is not an unreasonable interpretation since the HNE was used in this study is a racemic mixture of (4*R*)- and (4*S*)-hydroxynonenal.

In certain proteins, including glucose-6-phosphate dehydrogenase, lysine, but not histidine, might be a major target

amino acid of HNE. Lysine modification of proteins, particularly of low density lipoproteins (LDL), might be important in relation to the enhancement of atherogenicity by modified LDL (8–11). It has been noted that lysine modification on LDL generates immune complexes and accelerates macrophage uptake (32). Jürgens *et al.* (17) have demonstrated that loss of lysine residues is predominant in HNE-modified LDL and, to a lower extent, HNE attacks histidine residues. This emphasizes the importance of developing procedures that can be used to measure the amount of HNE-lysine adducts in LDL and other proteins.

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1. Winkler, P., Schaur, R. J. & Schauenstein, E. (1984) *Biochim. Biophys. Acta* **796**, 226–231.
2. Winkler, P., Linder, W., Esterbauer, H., Schauenstein, E. & Schaur, R. J. (1984) *Biochim. Biophys. Acta* **796**, 232–237.
3. Schauenstein, E. & Esterbauer, H. (1979) in *Submolecular Biological Cancer*, Ciba Found. Ser. (Excerpta Medica/Elsevier, Amsterdam), Vol. 67, pp. 225–244.
4. Esterbauer, H., Zollner, H. & Lang, J. (1985) *Biochem. J.* **228**, 363–373.
5. Esterbauer, H., Zollner, H. & Scholz, N. (1975) *Z. Naturforsch. BioSci.* **30c**, 466–473.
6. Esterbauer, H., Ertl, A. & Scholz, N. (1976) *Tetrahedron* **32**, 285–289.
7. Schauenstein, E., Taufer, M., Esterbauer, H., Kylianeck, A. & Seelich, T. (1971) *Monatsh Chem.* **102**, 517–529.
8. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. & Witztum, J. L. (1989) *N. Engl. J. Med.* **320**, 915–924.
9. Palinski, W., Yla-Herttuala, S., Rosenfeld, M. E., Butler, S., Socher, S. A., Parthasarathy, S., Curtiss, L. K. & Witztum, J. L. (1990) *Arteriosclerosis* **10**, 325–335.
10. Rosenfeld, M. E., Palinski, W., Yla-Herttuala, S., Butler, S. & Witztum, J. L. (1990) *Arteriosclerosis* **10**, 336–349.
11. 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.
12. Esterbauer, H., Schaur, R. J. & Zollner, H. (1991) *Free Radical Biol. Med.* **11**, 81–128.
13. Levine, R. L. (1983) *J. Biol. Chem.* **258**, 11823–11827.
14. Amici, A., Levine, R. L., Tsai, L. & Stadtman, E. R. (1989) *J. Biol. Chem.* **264**, 3341–3346.
15. Church, F. C., Porter, O. H., Catignani, G. L. & Swaisgood, G. L. (1985) *Anal. Biochem.* **146**, 343–348.
16. Crestfield, A. M., Moore, S. & Stein, W. H. (1963) *J. Biol. Chem.* **238**, 622–627.
17. Jürgens, G., Lang, J. & Esterbauer, H. (1986) *Biochim. Biophys. Acta* **875**, 103–114.
18. Hoff, H. F., O'Neil, J., Chisolm, G. M., III, Cole, T. B., Quehenberger, O., Esterbauer, H. & Jürgens, G. (1989) *Arteriosclerosis* **9**, 538–549.
19. Kaluza, Z., Chmielewski, M. & Pedersen, E. B. (1988) *Heterocycles* **27**, 1313–1316.
20. Benedetti, A., Comporti, M. & Esterbauer, H. (1980) *Biochim. Biophys. Acta* **620**, 281–296.
21. Uchida, K. & Stadtman, E. R. (1992) *Proc. Natl. Acad. Sci. USA*, in press.
22. Stadtman, E. R. (1990) *Biochemistry* **29**, 6323–6331.
23. Stadtman, E. R. (1990) *Free Radical Biol. Med.* **9**, 315–325.
24. Stadtman, E. R. & Oliver, C. N. (1991) *J. Biol. Chem.* **266**, 2005–2008.
25. Garrison, W. M., Jayko, M. E. & Bennett, W. (1962) *Radiat. Res.* **16**, 483–502.
26. Bateman, R. C., Jr., Youngblood, W. W., Busby, W. H. & Kizer, J. S. (1985) *J. Biol. Chem.* **260**, 9088–9091.
27. 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.
28. Starke-Reed, P. E. & Oliver, C. N. (1989) *Arch. Biochem. Biophys.* **275**, 559–567.
29. 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.
30. 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.
31. 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.
32. Haberland, M. E., Fogelman, A. M. & Edwards, P. A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1712–1716.