Evidence for a concerted reaction between lipid hydroperoxides and polypeptides

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ABSTRACT The events accompanying oxidative modification of low density lipoprotein (LDL) are multiple and complex, and the precise mechanisms remain to be determined. In the present studies, we examined a simple system in which we first prepared large amounts of lipid hydroperoxides (from linoleic acid or from phospholipids containing linoleic acid) by using soybean lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12). Linoleoyl hydroperoxide was then incubated with polypeptides in the absence of metal ions. We observed the generation of fluorescent products with a spectrum like that of oxidized LDL. The generation of fluorescent products from incubation of polypeptides with linoleoyl hydroperoxide was manyfold greater than that generated on incubation with preformed 4-hydroxynonenal at the same concentration. Superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) had no effect on the generation of fluorescent products. Incubation of linoleoyl hydroperoxide with cytochrome c (cyt c) under the same conditions led to progressive reduction of cyt c at a rate determined by the initial linoleoyl hydroperoxide concentration. This reduction was not significantly inhibited by probedal but was inhibited, although never completely, by superoxide dismutase. Even at 100 μg/ml, superoxide dismutase inhibited by only 65%. From these results, we are led to suggest a concerted reaction between the peroxo radical and free amino groups of polypeptides or phosphatidylethanolamine to generate fluorescent adducts. During oxidation of LDL or of cell membranes, this mechanism may occur side by side with the conventional Schiff base mechanism.

Oxidation of low density lipoprotein (LDL) converts it to a form that appears to be more atherogenic than native LDL in a number of different ways (1, 2). For example, oxidized LDL (but not native LDL) is recognized by macrophage scavenger receptors, leading to lipid accumulation. The protein component of the oxidized LDL appears to be centrally important in this process (3). At least three types of changes have been observed in apolipoprotein B subunit (apoB) of oxidized LDL: (i) a direct, oxidation-dependent fragmentation of the polypeptide chain (4); (ii) a decrease in the content of several amino acids, particularly lysine and histidine (4); and (iii) generation of fluorescence (5, 6). It has been generally assumed that the fluorescence is attributable to the formation of Schiff bases by conjugation of free aldehydes generated during peroxidative degradation of polyunsaturated fatty acids with amino groups (7). The fact that conjugation of LDL apoB with acetyl groups (8), with malondialdehyde (MDA) (9), or with any of several other aldehydes (5, 6) mimics some of the changes induced by oxidation is compatible with this interpretation, but the apoB conjugates have not been fully characterized and alternative mechanisms have not been ruled out.

Oxidation of the LDL particle is an almost bewilderingly complex process. It involves the apolipoprotein and the multiple lipid components of the particle; it appears to be dependent on an intrinsic phospholipase A2 activity (10); it ultimately results in breaking the peptide chain and conjugating lipids to proteins (6). Elucidation of mechanisms may therefore require the development of suitable simplified model systems. In the present studies, we have taken a step toward this by utilizing preformed lipid hydroperoxides (LOOHs) (generated from linoleic acid (18:2) or from linoleic acid—containing phospholipids) and incubating these with pure acceptor polypeptides in order to study the generation of fluorescent conjugates under controlled conditions. We have also studied the conjugation of phosphatidylethanolamine molecules with themselves during breakdown of phosphatidylethanolamine lipid peroxides. The results obtained suggest the possibility of unusual mechanisms involved in the interaction of hydroperoxides with proteins or phospholipids.

MATERIALS AND METHODS

Materials. Linoleic acid, 1-palmitoyl-2-linoleoyl 1-α-phosphatidylcholine, 1-palmitoyl-2-linoleoyl 1-α-phosphatidylylcholine, soybean lipoxygenase (SLO) type I and type V (linoleate:oxygen oxidoreductase, EC 1.13.11.12), superoxide dismutase (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1), polylsine (M, 3300), cupric diisopropylsalicylate (CuDIPS), and cytochrome c (cyt c) were purchased from Sigma. Arachidonic acid was from Calbiochem and CNBr-activated Sepharose 4B was from Pharmacia. N-Benzoyl leukmethylenelblue was from TCI America (Portland, OR) and [1-14C]linoleic acid was from DuPont NEN. 1-Palmitoyl 2-[1-14C]linoleoyl 1-α-phosphatidylethanolamine was prepared as reported for phosphatidylcholine (10). Coupling of SLO to Sepharose was performed according to the manufacturers instructions. MDA was freshly generated from malonaldehyde dimethylacetal by acid hydrolysis (11). 4-Hydroxynonenal (4-HNE) was kindly provided by Hermann Esterbauer (Graz, Austria). Hexanal was from Aldrich. α-(4-Pyridyl 1-oxide)-N-tert-butylnitrone and N-tert-butyl-α-phenylnitrone were provided by B. Kalyanaraman (Milwaukee, WI).

Lipoproteins. LDL (d = 1.019-1.063 g/ml) was isolated by preparative ultracentrifugation (12) from normal human plasma collected in EDTA (1 mg/ml). Protein was determined by the method of Lowry et al. (13) with bovine serum albumin (BSA) used as a standard.

Other Methods. Oxidation of linoleic and arachidonic acids by SLO was performed in phosphate-buffered saline (PBS), containing 0.3 mm EDTA at pH 7.4. Oxidation of phospho-

Abbreviations: LDL, low density lipoprotein; SOD, superoxide dismutase; cyt c, cytochrome c; SLO, soybean lipoxygenase; LOOH, lipid hydroperoxide; CuDIPS, cupric diisopropylsalicylate; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; BSA, bovine serum albumin.

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lipids was carried out in 0.1 M borate/10 mM deoxycholate buffer, pH 9.0. Lipid peroxidation was measured following the increase in absorbance at 234 nm with a UVikon 810 spectrophotometer (Kontron Instruments, Basel, Switzerland). The reduction of cyt c was followed by recording the change in absorbance between 500 and 600 nm. Cyt c was directly added to cuvettes containing lipid peroxides without prior extraction of the lipid. The amount of LOOH formed and of cyt c reduced was determined by using their respective molar extinction coefficients: 23 mM$^{-1}$cm$^{-1}$ at 234 nm for conjugated dienes and 21.1 mM$^{-1}$cm$^{-1}$ at 549 nm for reduced cyt c. LOOH was also measured by using leukomethylene-blue (14). The generation of fluorescent products was assayed with an LS50 Perkin-Elmer spectrofluorometer, again without extraction of the lipid peroxides.

Lipids were extracted with chloroform/methanol, separated on silica gel G TLC plates, and visualized with iodine vapor. Chloroform/methanol/water (65:25:4) was used for the separation of phospholipids and chloroform/methanol/water/acetate acid (90:10:0.5:0.5) was used for fatty acid products. $^{14}$C-labeled fatty acids and LOOH were scraped from the plates and radioactivity was determined.

RESULTS

Oxidation of Fatty Acids. Purified SLO type V was used to convert linoleic and arachidonic acids to the corresponding fatty acid hydroperoxides. The reaction was carried out in PBS at pH 7.4 and was usually complete within 1 hr as judged by the increase in absorbance at 234 nm. The addition of 0.3 mM EDTA to the reaction mixture did not inhibit the lipoxygenase reaction and EDTA was included unless otherwise specified. A typical incubation mixture (final vol, 1 ml) contained 100 nmol of fatty acid per ml (usually added to the reaction mixture in 2 μl of ethyl alcohol). The reaction was initiated by adding 100 units of lipoxygenase V unless otherwise indicated. The extent of the formation of LOOH was confirmed by thin-layer chromatography. Usually >75% of the added fatty acid was converted to LOOH in 60 min.

Generation of Fluorescent Conjugates from LOOH and Polypeptides. When LOOH (100 μM), prepared as described above, was subsequently incubated with lysine-containing peptides, fluorescent products were generated. As shown in Fig. 1A, polysyline yielded products with a fluorescence spectrum similar to that of oxidized LDL (Fig. 1B). Similar results were obtained with BSA (Fig. 1C). Unoxidized linoleic acid, used as a control, failed to generate fluorescence and when neither BSA nor polysyline was present no fluorescent product was formed. Qualitatively similar fluorescent derivatives were observed when the polypeptides were incubated with arachidonic acid hydroperoxide. The fluorescence spectrum of each of these products was similar to that of LDL oxidized in the presence of Cu$^{2+}$ (Fig. 1B), to that of oxidized LDL isolated from tissues (15), and also to that of 4-HNE-conjugated LDL (16). However, the fluorescence spectra of all of these differ considerably from the fluorescence spectrum of MDA-conjugated LDL (16).

The continued presence of lipoxygenase was shown not to be required for the generation of fluorescent products since lipid peroxides generated by using immobilized SLO, allowing for removal of the enzyme, which was then removed before addition of acceptor polypeptides, yielded similar results. Moreover, addition of SLO during the subsequent incubation of LOOH with polypeptides did not affect the rate of the reaction or the spectrum of the products.

If the formation of fluorescent conjugates depended on generating aldehydes from fragmentation of the linoleic acid, one would predict that an equimolar concentration of aldehydes instead of LOOH added at time 0 should be even more effective in generating such fluorescent compounds. However, when we replaced LOOH with 4-HNE or MDA at a concentration equal to that of the LOOH used (0.1 mM), there was almost no detectable formation of fluorescent compounds. Even at 0.5 mM, 4-HNE generated only ~72% of the fluorescence generated by incubation with 0.1 mM LOOH. The fluorescence generated from MDA showed a significantly different spectrum. We also tested hexanal, a monofunctional aldehyde. Coincubation of polysyline with hexanal did not generate fluorescent products even at a concentration of 5 mM.

Reduction of Cyt c by LOOH. It has been suggested that lipoxygenases can generate superoxide anion in the presence of a suitable cosubstrate, such as NADH (17). McNally et al. (18) have presented evidence that lipoxygenase-dependent generation of superoxide may mediate the oxidation of LDL by monocytes. To test the possibility that superoxide radicals might be generated in our system, cyt c was added after the initial 1-hr incubation of the fatty acids with lipoxygenase. There was significant reduction of cyt c over a 3-hr incubation (Fig. 2A), and the extent of cyt c reduction was dependent on the amount of LOOH present at the beginning of the incubation (Fig. 2B). In longer incubations (4 and 18 hr), 5.2 ± 0.7 and 14.3 ± 3.1 nmol of cyt c per 100 nmol of LOOH were reduced, respectively.

We considered the possibility that the continued presence of SLO carried over from the preparation of LOOH might play a role in the subsequent reduction of cyt c. It seemed unlikely since there was no obvious relationship between the concentration of SLO used and the subsequent reduction of cyt c—i.e., only the concentration of LOOH appeared to be determining. However, to definitively test for a role of SLO in the cyt c reduction step, we used immobilized SLO and

**Fig. 1.** Coincubation of LOOH with polysyline or BSA generates fluorescent products similar to those in oxidized LDL. LOOH was generated from linoleic acid (100 μg/ml) as described and then polysyline (100 μg/ml) was added (A), or BSA (100 μg/ml) was added (C). This mixture was incubated at room temperature for 18 hr. (B) Fluorescence of Cu$^{2+}$-oxidized LDL (10 μg/ml). Graphs show emission spectra. Excitation wavelength was 330 nm.
FIG. 2. Reduction of cyt c by LOOH. (A) Time course: 100 nmol of linoleic acid was treated with 100 units of SLO for 1 hr. Cyt c (100 µg/ml) was then added and the increase in absorption at 549 nm (reduced cyt c) was measured over 3 hr. The concentration of reduced cyt c was calculated as described. The result shown is representative of a number of repeated experiments. (B) Reduction of cyt c as a function of LOOH concentration. LOOH was prepared by incubating linoleic acid at a concentration of 50, 100, 400, and 600 µM with SLO as described. The LOOH preparations were then incubated with cyt c (100 µg/ml) for 3 hr. Graph shows the increase of absorbance at 549 nm, specific for reduced cyt c, at the end of the incubation.

were thus able to remove the enzyme before adding cyt c. The subsequent rate of cyt c reduction was unaffected. The mechanism of the cyt c reduction was explored. As shown in Table 1, neither a nonspecific protein (BSA) nor a radical scavenger (probucol) had a significant effect on the reduction of cyt c at the concentrations tested. In other experiments, catalase, two spin traps [α-(4-pyridyl 1-oxido)-N-tert-butyl-nitrone and N-tert-butyl-α-phenyl-nitrone], and mannitol were found to have no effect (data not shown). In contrast, the presence of SOD diminished the LOOH-dependent reduction of cyt c. The inhibition was small but significant at 1 µg/ml but was still incomplete even at SOD levels of 100 µg/ml (Table 2). In five studies with SOD at 100 µg/ml, the mean inhibition was 57.7% ± 8.3%. Both Cu/Zn-SOD and Mn-SOD were tested and gave similar results (data not shown). The nonprotein SOD analog CuDIPS also inhibited the reduction of cyt c, even more effectively than SOD itself (Table 1). The incomplete inhibition by SOD was shown not to be due to an inactivation of SOD by LOOHs. We treated 5 µg of SOD with 100 nmol of LOOH for 3 hr and then tested its ability to inhibit the reduction of cyt c by superoxide anion generated in the xanthine/xanthine oxidase system (19). The LOOH-treated SOD had retained fully its ability to inhibit the reduction of cyt c by the superoxide radicals generated in this system (60 nmol of superoxide anion per 15 min).

The limited inhibition by SOD and the high concentrations of enzyme needed raised questions about the identity of the cyt c reductant as being, in fact, the superoxide radical. We considered the possibility that SOD might have a direct effect on LOOH and that this would reduce the effective concentra-

| Table 1. Inhibition of LOOH-dependent cyt c reduction |
|---------------------------------|--------|----------|
| Cyt c reduced, nmol | % inhibition |
| LOOH | 3.46 | 61.5 |
| LOOH + SOD (100 µg/ml) | 1.45 | 58.1 |
| LOOH + CuDIPS (100 µg/ml) | 0.97 | 72.4 |
| LOOH + BSA (100 µg/ml) | 2.90 | 16.2 |
| LOOH + probucol (10 µM) | 3.05 | 11.8 |

First, 100 nmol of linoleic acid was treated with SLO for 1 hr. Then, SOD, CuDIPS, BSA, and probucol were added at the indicated concentrations. The reduction of cyt c (100 µg/ml) was then followed spectrophotometrically over 3 hr. The amount of reduced cyt c was calculated from the increase in absorption at 549 nm. Results shown are representative of two experiments.

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| Table 2. Inhibition of LOOH-dependent cyt c reduction by SOD |
|---------------------------------|--------|----------|
| Cyt c reduced, nmol | % inhibition |
| LOOH | 3.58 | 34.4 |
| LOOH + SOD (1 µg/ml) | 2.35 | 60.3 |
| LOOH + SOD (5 µg/ml) | 1.73 | 55.3 |
| LOOH + SOD (10 µg/ml) | 1.48 | 58.7 |
| LOOH + SOD (25 µg/ml) | 1.42 | 60.3 |
| LOOH + SOD (100 µg/ml) | 1.11 | 68.9 |

Linoleic acid-derived LOOH (100 nmol) was incubated with cyt c (100 µg/ml) and increasing concentrations of SOD for 3 hr. The increase in absorption at 549 nm was measured and used to calculate the amount of reduced cyt c. Representative results of two experiments are shown.

The inhibition of the operative cyt c reductant, regardless of whether it was LOOH itself or something derived from it. We measured the LOOH concentration directly (14) and found that SOD had no effect on the LOOH concentration remaining at the end of the incubation. We found that the original LOOH concentration fell by 30% at 21 hr regardless of whether or not SOD (100 µg/ml) was present. Another possibility considered was that the superoxide anion is generated in close proximity to the cytochrome and is therefore not readily available to the SOD. This, considered together with the greater effectiveness of LOOH in generating fluorescent derivatives compared to that of aldehydes, led us to conclude that a concert reaction of some kind is involved (Fig. 3). We suggest that some of the reduction of cyt c—that fraction inhibited by SOD—is due to generation of superoxide anion remote from the porphyrin iron. Another fraction—that not inhibited by SOD—is due to an interaction at a site on the cyt c protein very close to the iron. An implication of the reaction scheme proposed in Fig. 3 is that the full length of the fatty acid carbon chain would be initially attached to the protein moiety of cyt c, at least as an intermediate.

Covariant Linkage of [1-14C]Linoleyl Hydroperoxide to Cyt c. The possibility of a direct addition of LOOH to cyt c during LOOH-dependent reduction was tested. [14C]LOOH, prepared using [1-14C]linoleic acid and immobilized SLO, was incubated with cyt c (4 mg/ml) in the presence or absence of SOD (100 µg/ml). It was found that 14.3 ± 0.1 nmol of cyt c per 100 nmol of LOOH was reduced in 18 hr. It should be noted that in the same period of time the original LOOH concentration decreased by ~30%, a decrease of 30 nmol. The protein was then thoroughly separated by repeated precipitation with ice-cold acetone and assayed for 14C content. We found 14C radioactivity, corresponding to 3.63 ± 0.09 nmol of the initially added 100 nmol of [1-14C]LOOH, associated with cyt c, when no SOD was present during the incubation. The presence of SOD (100 µg) reduced the incorporation of 14C radioactivity into total proteins only minimally—to 2.95 ± 0.09 nmol of LOOH, of which the majority was linked to cyt c as determined by SDS/PAGE. This was confirmed in a separate incubation in which SOD alone had been incubated with [14C]LOOH. Only 0.45 ± 0.02 nmol of the added [1-14C]LOOH (100 nmol) was associated with SOD.

Role of SOD in Formation of a Fluorescent Compound. The hydroperoxide of linoleic acid (conversion to LOOH, >70%) was examined for formation of fluorescent adduct in the presence of polylysine. Concentrations of SOD (5 µg/ml) that were very effective in preventing the reduction of cyt c in the xanthine/xanthine oxidase system had no effect on the generation of fluorescent products, thus ruling out a specific role for superoxide anion in this system. However, high concentrations did cause a limited inhibition of the fluorescent product (18.8% ± 6.7%).

TheGeneration of Fluorescent Products from Oxidized Phosphatidylethanolamine. Oxidized phosphatidylethanolamine, containing a fatty acid hydroperoxide in the β position and a free
amino group, could act as both donor and acceptor. To test this possibility, we oxidized 1-palmitoyl 2-linoleoyl L-α-phosphatidylethanolamine in borate buffer (pH 9.0) in the presence of SLO and deoxycholate. The generation of oxidized product was assessed by following the increase in absorption at 234 nm. After 1 hr, the pH of the incubation mixture was lowered to 7.4 and the sample was transferred to the fluorescence spectrophotometer. We observed the progressive formation of a fluorescent product with excitation/emission maxima at 350 and 420 nm, respectively (Fig. 4), exactly like that of oxidized LDL (Fig. 1B). When 1-palmitoyl 2-linoleoyl L-α-phosphatidylethanolamine was treated in exactly the same way, no fluorescent product could be demonstrated, presumably because of the absence of a free amino group acceptor. We also tried to generate fluorescent adduct by coincubating phosphatidylethanolamine with various aldehydes: MDA, 4-HNE, and hexanal. We could not show any fluorescent adduct when the phosphatidylethanolamine and the aldehydes were incubated at a molar ratio of 1:1.

**DISCUSSION**

The first important conclusion that we draw from these studies is that generation of fluorescent products from the interaction of LOOH with polylysine or BSA is very unlikely to be attributable to generation of free aldehydes and their subsequent conjugation to the polypeptide. Using the same molar concentration of aldehyde and LOOH, the yield of fluorescent compound from preformed aldehyde (4-HNE) was <15% of the yield from LOOH. In view of the fact that only 30% of the LOOH initially present was degraded during the incubation, and that in any case only some small portion of that would yield reactive aldehydes, the disparity becomes even greater. These findings strongly suggest an alternative reaction mechanism. Esterbauer et al. (20) have called attention to the fact that the concentration of aldehydes generated during oxidation of LDL, if the aldehydes were distributed throughout the aqueous phase, would be much lower than that needed to generate fluorescence comparable to the fluorescence actually generated during the oxidation of LDL. In the case of LDL, it can be argued that the aldehydes—at least the long-chain aldehydes—remain in the lipid phase and are, therefore, present at a high chemical activity within the LDL particle. Esterbauer and colleagues (21) have calculated that the concentration of 4-HNE in the lipid phase of oxidized LDL might be as high as 10–50 mM and might thus account for the extent of conjugation observed. In the present studies, however, there is no lipid phase to sequester the aldehydes and this sort of explanation does not apply. Therefore, we had to look to alternative reaction mechanisms.

We suggest two possible reaction pathways. Both are initiated by interaction of a free amino group with the peroxyl radical. The first, as shown in Fig. 3 (pathway A), involves a release of superoxide anion concurrent with generation of the covalent linkage of linoleate to the protein. The superoxide released could account for the reduction of cyt c and the ability of SOD to inhibit it. There might then be a second oxidative step leading to cleavage at the 9 position, releasing the first 9-carbon fragment of the linoleic acid, after which there could be Schiff base formation, either with a second amino group on the same protein molecule or with an amino group on a second protein molecule, crosslinking two molecules of the protein. An alternative pathway involves a concerted reaction in which the peroxyl radical at C-13 immediately oxidizes at the 9,10 position, leading directly to a fluorescent closed-ring structure with the release of the first 9 carbons from the linoleic acid (pathway A). Another possibility (pathway B) is that the nucleophilic displacement.

**FIG. 3.** Suggested pathways (A and B) for the concerted reaction suggested by the data presented in this paper.

**FIG. 4.** Fluorescence spectra of SLO-treated phosphatidylethanolamine. Phosphatidylethanolamine (100 nmol/ml) was treated with SLO as described. Fluorescence spectra were recorded after incubation for 15 hr at room temperature. Spectra obtained show excitation maximum at 350 nm and emission maximum at 420 nm.
of the peroxy radical at C-13 is accompanied by concurrent oxidation of the $\Delta^2\Delta^3$ double bond to generate an epoxy ring at $\Delta^2\Delta^3$. This in turn can react with the imino group, leading to formation of either a 5- or a 6-membered ring.

We do not by any means suggest that these are the exclusive mechanisms operative, even in this highly simplified system, and, in LDL or in complex biological membranes, there are undoubtedly many different operative pathways. Much of the degradation of polyunsaturated fatty acids to smaller fragments occurs independently of conjugation to the protein moiety. The interesting proposition here is that the formation of fluorescent conjugates involves a direct interaction of the liperoxyl radical with amino groups (either in the protein or in phosphatidylethanolamine) without prior fragmentation to yield aldehydes or other stable end products.

The mechanisms proposed can account for most of the findings reported here: (i) the generation of fluorescent products; (ii) the generation of superoxide anion (although possibly only from a fraction of the LOOH molecules degraded); (iii) the covalent linkage of the carboxyl carbon of [1-14C]linoleoyl hydroperoxide to protein, also observed by Steinbrecher (6); (iv) the failure of SOD to significantly decrease the covalent bonding of the acyl chain to protein or to affect the formation of fluorescent products; and (v) the reduction of cyt c with only partial inhibition by SOD. Because some of the superoxide radical is postulated to be generated at a site on the cyt c protein very near the heme, SOD cannot have easy access to it. Indeed, the somewhat greater effectiveness of CuDIPS may reflect the fact that its lower molecular weight gives it better access to the site of generation of superoxide anion.

The extent to which the mechanisms suggested here apply during oxidation of LDL and other lipid/protein mixtures can only be determined by additional studies. One finding that favors this or some related mechanism is the fact that the amino acid composition of LDL changes in a fairly predictable fashion in the course of LDL oxidation. The lysine content decreases by $\approx 15\%$ and the histidine content decreases by $\approx 32\%$, while the aspartic acid content increases by a little over 5% (4). This is in contrast to what happens when LDL is conjugated with 4-HNE (or, presumably, with other aldehydes). After acid hydrolysis there is no apparent loss of lysine residues because acid hydrolysis regenerates the lysine from its Schiff base conjugates (21). The possibility must be kept in mind, however, that the Schiff bases formed initially may undergo rearrangements or modifications in structure such that acid hydrolysis does not restore intact lysine. Steinbrecher (6) reported that during 20 hr of Cu-induced oxidation the number of lysine residues reactive with trinitrobenzenesulfonic acid decreased by $\approx 40\%$. Amino acid analysis, however, showed only a 17% decrease in the number of intact lysine residues. Most of the difference could be accounted for by residues nonreactive with dinitrofluorobenzene but yielding lysine on acid hydrolysis—i.e., Schiff bases. Thus, it would seem that in the case of LDL oxidation at least two different mechanisms may be operative—one reversible (Schiff base formation) and one irreversible—by the mechanism proposed here or by related mechanism(s).

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