Structural characterization of the products of hydroxyl-radical damage to leucine and their detection on proteins

Shan-Lin FU* and Roger T. DEAN

Heart Research Institute, 145 Missenden Road, Camperdown, NSW 2050, Australia

We have previously reported the formation of valine hydroperoxides and aldehydes from hydroxyl-radical attack on free valine and protein molecules. We have also demonstrated that the major degradation products of valine hydroperoxides by several biochemical and cellular systems are the corresponding hydroxides, and therefore proposed that hydroxyvalines may serve as useful *in vivo* markers for studying protein oxidation. Here we have undertaken the structural elucidation of the oxidation products of leucine, another amino acid which is very susceptible to peroxidation. Hydroxyl-radical ($HO^{\bullet}$) attack on L-leucine in the presence of oxygen, followed by $NaBH_4$ reduction, gave rise to five major oxidation products which have been isolated and identified. On the basis of chemical and spectroscopic evidence, the five products have been identified as (2*S*)-γ-hydroxyleucine, (2*S*,4*S*)-δ-hydroxyleucine, (2*S*,4*R*)-δ-hydroxyleucine, (2*S*,4*R*)-4-methylproline (*trans*-4-methyl-L-proline) and (2*S*,4*S*)-4-methylproline (*cis*-4-methyl-L-proline). The three hydroxyleucines have been confirmed to be the reduction products of the corresponding hydroperoxyleucines, while the two proline analogues are from reduction of their corresponding cyclic Schiff bases. By HPLC analysis using post-column *o*-phthaldialdehyde derivatization, we have detected hydroxyleucines in the hydrolysates of tripeptides and proteins which had been γ-radiolysed and treated with $NaBH_4$. Furthermore, we demonstrate the occurrence of the hydroxyleucines on proteins in physiological and pathological samples. Hydroxyleucines, like hydroxyvalines, may provide useful *in vivo* markers for studying protein oxidation. In the present study we also investigated the competition between leucine, valine and phenylalanine for $HO^{\bullet}$, and proposed a possible radical-transfer process in such free-radical reactions.

INTRODUCTION

Free radicals are inevitable by-products of biological redox reactions (reviewed in [1,2]). Free-radical–protein interactions are now believed to be closely related to the physiology of aging and to pathologies such as atherosclerosis [1,3,4]. Protein damage by free-radical exposure induces cross-linking and backbone fragmentation, as well as amino acid modification. The latter give rise to carbonyl formation [5], protein-bound dopa (3,4-dihydroxyphenylalanine) [6,7] and protein hydroperoxides [8], which, on reaction with transition metals, produce several secondary reactive radical species which have been detected by ESR spin trapping [9].

In the course of searching for a suitable marker for studying protein oxidation, we have previously focused on the oxidation products of valine generated by free-radical damage, as valine is one of the free amino acids on which hydroperoxides are most readily found during hydroxyl-radical ($HO^{\bullet}$) attack [8–10]. We have isolated and identified three valine hydroperoxides (β-hydroperoxyvaline, (2*S*,3*S*)-γ-hydroperoxyvaline and (2*S*,3*R*)-γ-hydroperoxyvaline) from radiolysis of valine, peptide and protein solutions in the presence of oxygen [11]. We have also demonstrated that valine hydroxides are the major biological degradation products of valine hydroperoxides on free valine, peptides and proteins in the presence of: (1) transition metals (Fe^{2+}, Fe^{3+} or Cu^{2+}); (2) the detoxifying enzyme glutathione peroxidase; (3) human plasma; and (4) J774 mouse macrophage-like cells [12]. Following the successful detection of hydroxyvaline in atherosclerotic plaque samples and native LDLs (low-density lipoproteins) (S.-L. Fu and R. T. Dean, unpublished work), we envisage that valine hydroxide may be a useful marker for studying protein oxidation *in vivo*.

Both iodometric assays [10] and ESR experiments [9] demonstrate that generation of hydroperoxyleucines from radiolysis of leucine in the presence of oxygen is also substantial. Indeed, a body of literature reports significant loss of leucine after $HO^{\bullet}$ attack on several proteins (reviewed in [13]). Furthermore, the reaction rate of $HO^{\bullet}$ with leucine at neutral pH is approx. 3-fold higher than that with valine [14]. We therefore studied further the oxidation of leucine by $HO^{\bullet}$ and applied the knowledge gained to protein oxidation. In the present paper we report results from such structural studies and discuss a possible radical transfer between leucine and other amino acids. We demonstrate the occurence of hydroxyleucines in normal human plasma and LDL and their elevation in cataractous as against normal lens proteins.

MATERIALS AND METHODS

Water used was filtered through a four-stage Milli Q system (Millipore-Waters, Lane Cove, NSW, Australia) equipped with a 0.2 μm-pore-size final filter. Amino acids, Ala-Val-Leu, BSA (fatty-acid-free), *o*-phthaldialdehyde (OPA) reagent solution (incomplete), isoluminol (6-amino-2,3-dihydrophthalazine-1,4-dione) and microperoxidase (MP11) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2-Mercaptoethanol and mercaptoacetic acid were from Merck (Kilsyth, VIC, Australia). Other chemicals, solvents and chromatographic materials were of AR or HPLC grade.

Abbreviations used: OPA, *o*-phthaldialdehyde; dopa, 3,4-dihydroxyphenylalanine; LDL, low-density lipoprotein; EI, electron impact; ES, electrospray; $HO^{\bullet}$, hydroxyl radical; Leu.OH1, (2*S*)-γ-hydroxyleucine; Leu.OH2,3, δ-hydroxyleucines; Leu.4, (2*S*,4*R*)-4-methylproline; Leu.5, (2*S*,4*S*)-4-methylproline.

* To whom correspondence should be addressed.

Human LDL [relative density (d) 1.021–1.063] was prepared by density-gradient ultracentrifugation of plasma from normal fasting donors [15]. Protein content of LDL was determined using bicinchoninic acid method (Sigma) with BSA as standard. Absorbance at 562 nm was measured after incubation for 1 h at 60 °C.

Three normal and three cataractous human lenses were kindly given by Professor Roger J. W. Truscott, Australian Cataract Research Foundation, University of Wollongong, Wollongong, NSW, Australia. The three cataract lenses were classified as type IV on the basis of the degree of nuclear browning as described by Pirie [16]. Normal lenses were stored at −80 °C within 24 h of death, and cataractous lenses were stored immediately upon excision.

Amino acids, peptides and proteins were oxidized by $HO^{\bullet}$ generated from ^{60}Co radiolysis of water in the presence of oxygen, as previously described [8,11]. The absolute quantity of hydroperoxides generated from radiolysis was determined by using an iodometric assay [11]. Hydroperoxides generated from γ-radiolysis of leucine were also detected by using the chemiluminescence assay [17–19], which involves an HPLC separation on an LC-NH_2 column (25 cm × 4.6 mm; 5 μm particle size; Supelco, Bellefonte, PA, U.S.A.) and post-column reaction with a reagent containing microperoxidase and isoluminol as described previously [11]. The method developed to obtain the best recovery for dopa from protein hydrolysis was adopted [6]; this was gas-phase hydrolysis, and utilized 5 % (v/v) mercaptoacetic acid and 1 % (w/v) phenol in 6 M HCl solution as reductants. For plasma and LDL samples, proteins were purified by trichloroacetic acid precipitation following acetone and diethyl ether washing [20] before hydrolysis. For the cataractous-lens samples, the frozen materials were powdered and freeze-dried before hydrolysis. To avoid loss of hydroperoxides during hydrolysis, samples containing hydroperoxides were pre-reduced by $NaBH_4$ treatment (1 mg/ml), as described previously [11].

MS and NMR

Electrospray (ES) MS was conducted on a VG Platform mass spectrometer (Fisons, Homebush, NSW, Australia). The samples (~ 100 pmol/μl) were dissolved in aq. 50 % acetonitrile. Formic acid (0.5 %) was added to the solutions to assist with protonation of the solute.The solvent was delivered by a Phoenix (Fisons) syringe pump at a flow rate of 5 μl/min; 10 μl of each solution was injected for analysis. A dry nitrogen bath gas at atmospheric pressure was employed to assist evaporation of the electrospray droplets. The electrospray probe tip potential was 3.5 kV with 0.5 kV on the chicane counter-electrode. A sample cone potential of 15 V was used.

Electron impact (EI) mass spectra were recorded on a Finnigan (San Jose, CA, U.S.A.) Mat TSQ 46 quadrupole analyser. Ionization involved 70 eV electron energy. The samples were introduced on a desorption probe with source temperature of 140 °C.

NMR studies were performed on a Varian Gemini 300 BB spectrometer. Samples were dissolved in ^2H_2O containing 0.01 M ^2HCl. For proton (^1H-) NMR (300 MHz), the chemical shifts recorded were referred to the non- or partially ^2H-labelled water signal, which was set to δ 4.76 p.p.m.. For the ^{13}C-NMR (75 MHz) study, chemical shifts measured were referred externally to the carbon chemical shift of p-dioxan, which was set to δ 66.5 p.p.m. Signal assignment was based on spectral data from both distortionless enhancement by polarization transfer ('DEPT') [21] and fully proton-decoupled experiments.

HPLC analysis of OPA derivatives of amino acids (OPA–HPLC method)

Derivatization of amino acids with OPA reagent in the presence of 2-mercaptoethanol (0.5 %, v/v) was automated using an SIL-10A autosampler (Shimadzu, South Rydalmere, NSW, Australia) as described previously [11]. HPLC analysis was carried out on a LC-10A HPLC system (Shimadzu) fitted with a high-pressure gradient mixer. The OPA derivatives of amino acids were separated on a Zorbax ODS column (25 cm × 4.6 mm; 5 μm particle size, Rockland Technologies, Newport, DE, U.S.A.) with a Pelliguard column (2 cm; Supelco) eluted with a gradient of solvent A [methanol/tetrahydrofuran/20 mM sodium acetate, pH 5.2 (8:1:31, by vol.)] and solvent B [same solvents (32:1:7, by vol.]. The gradient was generated as follows: 0 % B to 25 % B in 8 min; isocratic elution for 5 min; then to 40 % B in 10 min; then to 50 % in 2 min; isocratic elution for 6 min, then to 100 % B in 4 min; isocratic elution for 3 min; then re-equilibration at 0 % B for 12 min for next analysis. The flow rate was 1 ml/min and the eluent was monitored by a Hitachi F-1080 fluorescence spectrometer with an excitation wavelength at 340 nm and an emission wavelength at 440 nm. A modification from our previous method [11] was the use of a column heater at 30 °C. Data were analysed by Class LC-10 software (Shimadzu).

RESULTS AND DISCUSSION

Leucine oxidation by $HO^{\bullet}$ in the presence of oxygen

Radiolysis (^{60}Co, 1200 Gy) of L-leucine (1 mM in water) in an oxygen-saturated system produces leucine hydroperoxides at a concentration of 90 μM, determined by iodometry. The formation of leucine hydroperoxides was also studied using post-column chemiluminescence detection (Figure 1a). The three chemiluminescent peaks, which were eluted at 12.9, 14.7 and 16.0 min respectively, were due to hydroperoxides, as they were completely

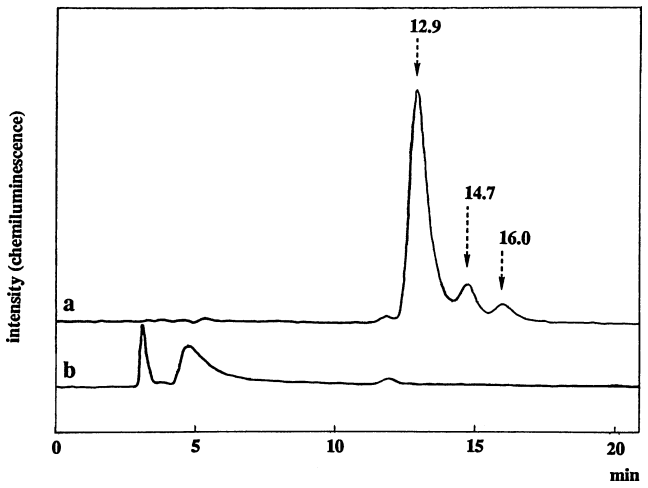


Figure 1 HPLC post-column chemiluminescence detection of leucine hydroperoxides generated during ^{60}Co radiolysis of leucine (1 mM in water) in the presence of oxygen

Trace a, before $NaBH_4$ reduction; trace b, after $NaBH_4$ reduction. HPLC was performed on an analytical LC-NH_2 column (25 cm × 4.6 mm; 5 μm particle size, Supelco) with 81 % acetonitrile in 10 mM sodium phosphate, pH 4.3, as the mobile phase, elution being at a rate of 1.0 ml/min. Post-column chemiluminescence reagent was delivered at 1.5 ml/min. The reaction mixture was monitored using a CLD-110 chemiluminescence detector (Tohoku, Rifu cho. Miyagi, Japan).

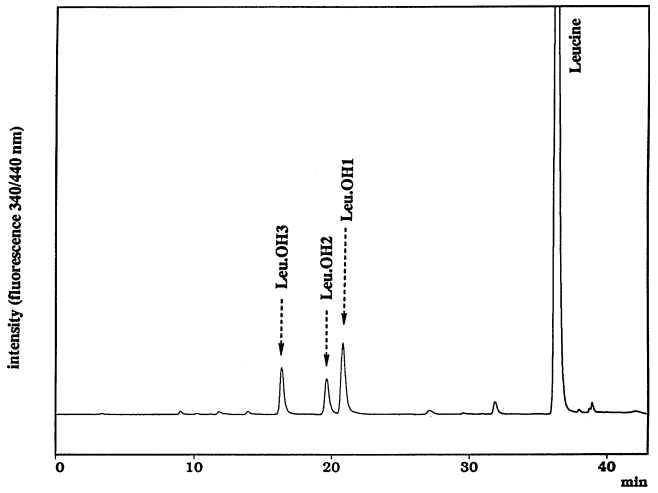


Figure 2 HPLC detection of OPA derivatives of leucine hydroxides

γ-Radiolysed leucine solution (1 mM) was diluted 20-fold before subjected to OPA-HPLC analysis (see the Materials and methods section).

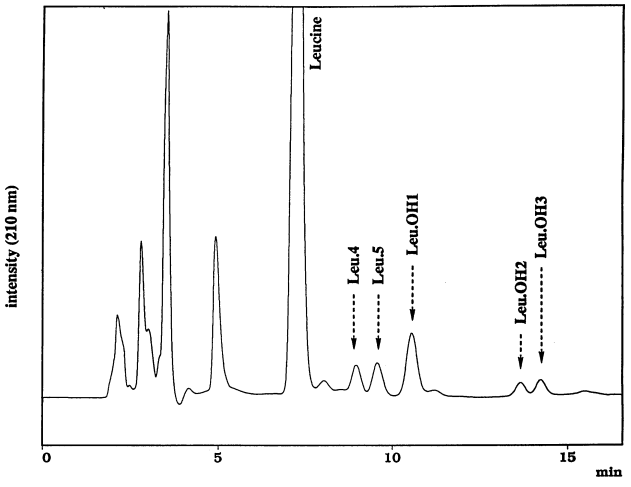
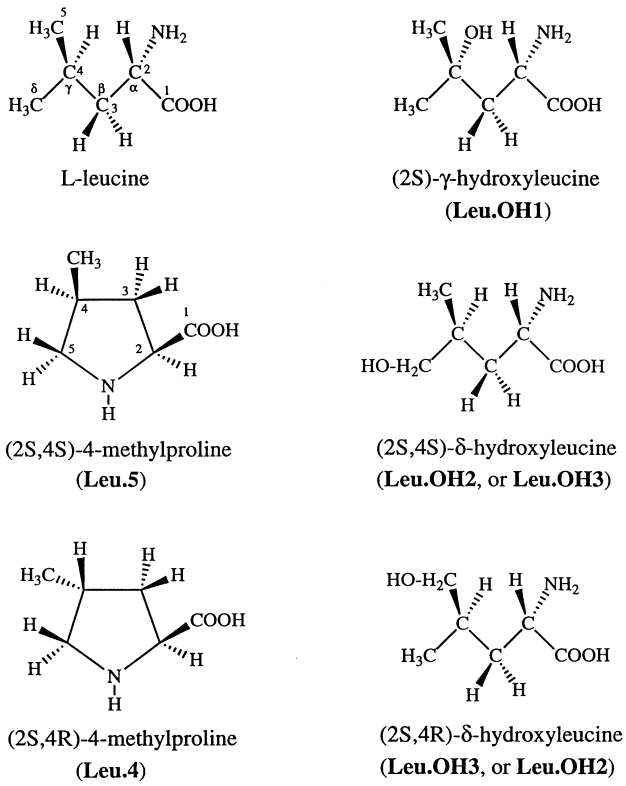


Figure 3 HPLC of γ-radiolysed leucine followed by reduction by $NaBH_4$

A γ-radiolysed leucine solution (1 mM) was treated with $NaBH_4$ (1 mg/ml) and then concentrated 40-fold under decreased pressure. HPLC analysis of the sample (100 μl) was performed using a semi-preparative LC-NH_2 column (25 cm $\times$ 10 mm; 5 μm particle size; Supelco) with 81% acetonitrile in 10 mM sodium phosphate, pH 4.3, as the mobile phase, elution being at a rate of 5.0 ml/min and with monitoring at 210 nm.



Scheme 1 Structures of leucine and its oxidation products

removed after reduction of the sample with $NaBH_4$ (Figure 1b) [11,18].

Since 2-mercaptoethanol present in the OPA reagent reduces amino acid hydroperoxides to hydroxides [11,12], hydroxyleucine is expected to be formed during OPA derivatization of hydroperoxyleucine. Indeed, OPA-HPLC analysis of the γ-radiolysed leucine revealed three significant peaks of oxidized leucine, later identified as hydroxyleucine: Leu.OH1 (20.8 min), Leu.OH2 (19.6 min), and Leu.OH3 (16.3 min) (Figure 2). These were well separated from other amino acids, including hydroxyvaline [cf. β-hydroxyvaline (Val.OH1) 21.5 min, (2*S*,3*S*)-γ-hydroxyvaline (Val.OH2) 17.9 min and (2*S*,3*R*)-γ-hydroxyvaline (Val.OH3) 12.2 min; results not shown]. It has been demonstrated clearly that the OPA derivatives of hydroxyvaline have similar fluorescence yields to that of valine [11]. By analogy, it can be expected that the same will apply to leucine and hydroxyleucine. Thus a total yield of 92 μM hydroxyleucine (61 μM Leu.OH1, 15 μM Leu.OH2 and 16 μM Leu.OH3) was determined from the OPA-HPLC assay by using a standard curve of leucine for calibration of hydroxyleucine, and this corresponds well with the hydroperoxyleucine value (90 μM) from the iodometric assay. The close correlation confirms that the OPA derivatives of hydroxyleucine have similar fluorescence yields to that of the parent amino acid. $NaBH_4$ reduction of the same sample prior to OPA-HPLC analysis did not increase the yield of hydroxyleucine, indicating that hydroperoxyleucine was the sole precursor for hydroxyleucine. This is different from the situation for valine, where valine aldehyde was formed from radiolysis and contributed to the formation of γ-hydroxyvaline on reduction by $NaBH_4$ [11], unlike the situation with leucine aldehyde discussed below.

Separation and structural identification of oxidation products of leucine

For practical reasons, attempts to isolate and purify oxidized leucine molecules for structural determination were made only after the samples were treated with $NaBH_4$. To obtain enough material for structural work, a large-scale γ-irradiation (1200 Gy) of leucine solution (100 ml, 1 mM) was conducted. After reduction with $NaBH_4$ (10 mg) and concentration 100-fold under decreased pressure, the mixture was separated by HPLC on a semi-preparative LC-NH_2 column with UV detection at 210 nm. A typical HPLC profile is shown in Figure 3. The five fractions, designated Leu.OH1, Leu.OH2, Leu.OH3, Leu.4 and Leu.5 respectively, were collected from ten separate injections. After

Table 1 ES-MS and EI-MS data of oxidized products of leucine and their related amino acids

Protonated molecular ions (*M*H) were recorded by using ES-MS. Fragment ions (*m*/*z*) were recorded by using EI-MS with their intensity relative to the most intensive ion peak (base peak) in parentheses. Detailed experimental conditions were described in the Materials and methods section. Abbreviation: *trans*-4-HO-L-Pro, *trans*-4-hydroxy-L-proline. EI-MS analysis was not performed on Leu.OH1, 2 and 3.

	ES-MS *M*H	EI-MS (*m*/*z*) M^+	$M-45$	Other fragments
Leu.OH1	148			
Leu.OH2	148			
Leu.OH3	148			
Leu.4	130	129 (2.5%)	84 (100%)	69 (30%, $M-45-15$)
Leu.5	130	129 (3.0%)	84 (100%)	69 (35%, $M-45-15$)
Leucine	132	131 (0.1%)	86 (100%)	74 (45%, $M-57$)
Proline	116	115 (2.0%)	70 (100%)	57 (12%, $M-74$)
trans-4-HO-L-Pro	132	131 (1.5%)	86 (100%)	68 (65%, $M-45-18$)

concentration, the fractions were individually desalted and further purified on the same column with acetonitrile/water (4:1, v/v) containing 0.005% trifluoroacetic acid as the mobile phase, before MS and NMR studies.

On the basis of ES-MS, ^{1}H-NMR, ^{13}C-NMR data and chemical reactions, the chemical structures of the five oxidation products of L-leucine were determined as (2*S*)-γ-hydroxyleucine (Leu.OH1), (2*S*,4*S*)-δ-hydroxyleucine (Leu.OH2 or Leu.OH3), (2*S*,4*R*)-δ-hydroxyleucine (Leu.OH3, or Leu.OH2), (2*S*,4*R*)-4-methylproline (Leu.4) and (2*S*,4*S*)-4-methylproline (Leu.5). Their structures are shown in Scheme 1.

Hydroxyleucine residues

The nature of the three hydroxyleucine residues was revealed from post-column chemiluminescence detection of their hydroperoxide precursors and OPA-HPLC analysis. ES-MS studies (Table 1) provided the same molecular mass of 147 for the three hydroxyleucines, indicating that they differ from each other only in the position of the hydroxy group.

Table 3 ^{13}C chemical shifts of oxidized products of leucine and their related amino acids

Samples were dissolved in 0.01 M ^{2}HCl in ^2H_2O. Chemical shifts (δ, in p.p.m.) were referenced externally to the carbon signal of *p*-dioxan, which was set at 66.5 p.p.m.. Signal assignment was based on spectral data from both DEPT (see the text) and fully proton-decoupled experiments. *trans*- or *cis*-4-HO-L-Pro stands for *trans*- or *cis*-4-hydroxy-L-proline.

	C-1 (>**C**=O)	**C**-2 (Cα)	**C**-3 (Cβ)	**C**-4 (Cγ)	**C**-5 (Cδ)	4-**C**H$_3$
L-Leucine	175.0	53.1	39.7	24.2	–	21.0, 22.0
Leu.OH1	176.5	52.9	41.5	68.1	–	28.9, 30.8
Leu.OH2	175.5	54.2	36.9	34.0	68.4	18.3
Leu.OH3	175.0	54.5	37.0	34.5	69.1	18.1
Leu.4	174.8	61.4	37.4	32.9	53.3	17.0
Leu.5	175.0	62.2	38.1	34.7	53.6	17.4
trans-4-HO-L-Pro	174.1	59.6	37.3	69.9	52.8	–
cis-4-HO-L-Pro	174.4	59.7	37.2	69.2	53.1	–

Clear evidence leading to the structural determination of the three hydroxyleucine came from both ^{1}H-NMR and ^{13}C-NMR studies summarized in Tables 2 and 3. For Leu.OH1, the protons of the two methyl groups resonate as two singlets (Table 2), indicating that the γ-proton in leucine is replaced by a hydroxy group. The downfield shifts of the methyl protons in Leu.OH1 (δ 1.27 p.p.m. and δ 1.28 p.p.m.), versus δ 0.89 p.p.m. and δ 0.90 p.p.m. in leucine, are due to the withdrawing effect of the adjacent hydroxy group. A deshielding effect of the hydroxy group can also be observed from the chemical shifts of its adjacent carbons (Table 3). The γ-carbon, with the hydroxy group directly attached to it, experiences the greatest deshielding effect and resonates at δ 68.1 p.p.m., over 40 p.p.m. downfield from the γ-carbon in leucine (δ 24.2 p.p.m.). The two methyl carbon atoms in Leu.OH1 resonate at δ 28.9 p.p.m. and δ 30.8 p.p.m., about 10 p.p.m. downfield from their counterparts in leucine (δ 21.0 p.p.m. and δ 22.0 p.p.m.), due to the 'β-effect' of the oxygen atom. The values are close to those of the analogous carbon atoms in β-hydroxyvaline (δ 25.9 p.p.m. and δ

Table 2 Proton chemical shifts of oxidized products of leucine and their related amino acids

Samples were dissolved in 0.01 M ^{2}HCl in ^2H_2O. Chemical shifts (δ, in p.p.m.) were referenced to the non- or partially ^{2}H-labelled water signal, which was set to 4.76 p.p.m. Signals were expressed as, 's' for singlet, 'd' for doublet, and 'm' for multiplet. Values in parentheses are the coupling constants in Hz. Signal assignment for Leu.4 and Leu.5 was performed with the aid of a ^{1}H–^{1}H COSY experiment.

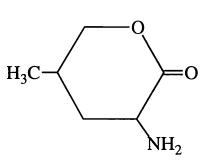
	2-H (Hα)	3-H$_a$ (Hβ)	3-H$_b$ (Hβ)	4-H	4-C**H**$_3$	4-C**H**$_2$
L-Leucine	3.72 m	←	1.57–1.74 m	→	0.89 d (5.5) 0.90 d (5.4)	—
Leu.OH1	4.13 m	1.92 dd (15.2, 10.1)	2.12 dd (15.2, 3.5)	—	1.27 s 1.28 s	—
Leu.OH2	4.00 m	1.79 m	1.97 m	1.64 m	0.91 d (6.6)	3.40 dd (11.2, 5.2) 3.45 dd (11.2, 6.2)
Leu.OH3	4.00 m	←	1.71–1.88 m	→	0.92 d (5.7)	3.42 dd (11.0, 5.2) 3.45 dd (11.0, 5.7)
Leu.4	4.32 dd (9.6, 4.3)	1.96 ddd (13.6, 9.6, 8.9)	2.29 m	2.38 m	1.05 d (6.6)	2.87 dd (11.2, 8.9) 3.54 dd (11.2, 6.9)
Leu.5	4.21 dd (9.0, 9.0)	1.63 ddd (13.0, 9.5, 9.0)	2.50 m	2.40 m	1.02 d (6.6)	2.89 dd (11.0, 11.0) 3.44 dd (11.0, 7.4)

30.0 p.p.m.; see [11]). Leu.OH1 is thus identified as (2*S*)-γ-hydroxyleucine (γ-hydroxy-L-leucine).

For Leu.OH2 there is only one methyl proton signal in the ^{1}H-NMR spectrum, resonating at δ 0.91 p.p.m. as a doublet with a coupling constant of 6.6 Hz (instead of two in leucine) (Table 2), indicating that a hydroxy group is at one of the methyl carbon atoms in leucine. The two methylene protons in the resultant $-CH_2OH$ group resonate at low field (δ 3.40 p.p.m. and 3.45 p.p.m.), each as doublets of doublets. The magnetic non-equivalence of these two protons is due to the creation of a new chiral centre at the γ-carbon. Due to the 'β-effect' of the oxygen atom in the –OH group, the methylene carbon resonates at δ 68.4 p.p.m., shifted over 40 p.p.m. downfield compared with the methyls in leucine. The methyl carbon (at δ 18.3 p.p.m.) shifted about 4 p.p.m. upfield from its counterparts in leucine (Table 3) due to a steric 'γ-interaction' [22] from the hydroxy oxygen. Leu.OH3 gives very similar NMR data to Leu.OH2. The two are thus diastereomers differing only at C-4. They are identified as (2*S*,4*S*)- and (2*S*,4*R*)-δ-hydroxyleucine, but we have not determined which is Leu.OH2 or 3.

4-Methylproline residues

ES-MS analysis showed that both Leu.4 and Leu.5 have a molecular mass of 129, 2 mass units less than leucine (Table 1). ^{1}H-NMR and ^{13}C-NMR data revealed their similarities with leucine. There are a total of six carbon signals, including one methyl carbon atom (at about δ 17 p.p.m.) and one carboxy carbon atom (at about δ 175 p.p.m.; Table 3). We observed one methyl proton signal at about δ 1 p.p.m. as a doublet and another six proton signals ranging from δ 1.6 to 4.3 p.p.m. (Table 2). No vinyl proton signals were observed in the ^{1}H-NMR spectra, indicating that there is no additional double bond. On the basis of these data, there are only two possible structures: δ-lactones (I) or proline analogues (II). In any case the acyclic leucine is cyclized, either via the carboxy group to form compound (I) or via the amino group to form compound (II). ^{1}H-NMR data fit reasonably well with both proposed structures, although ^{13}C-NMR data favour the latter. In particular, the carbon signal at δ 53.3 p.p.m. is rather low to represent the δ-carbon in the lactone (compound I), which is expected to be around δ 68 p.p.m., but appropriate for the δ-carbon in 4-methyl-L-proline (compound II). This argument is supported by the close similarity of the carbon-13 chemical shifts for Leu.4/5 to those for 4-hydroxyproline (Table 3).



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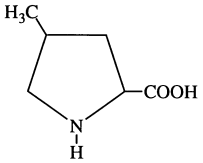


II

EI-MS studies showed that loss of -COOH is a characteristic fragmentation for amino acids such as leucine, valine, proline and 4-hydroxyproline, giving a very weak molecular ion (*M*) ($< 5\%$) and a base fragment ion $M-45$ (100%) (Table 1). The observed base ion for Leu.4/5 at m/z 84 (129 − 45) with a very weak molecular ion at m/z 129 ($< 3\%$) indicates that the carboxy group is retained, unlike the situation with the lactone.

Chemical evidence to support the identification of Leu.4/5 as 4-methylproline came from their reactivity towards NaOH and OPA reagent. Reaction of Leu.4/5 with 2 M NaOH at 60 °C for 1 h failed to produce the expected δ-hydroxyleucine residues, which precludes their being lactones. In addition, Leu.4 and Leu.5, like proline, do not form OPA derivatives, so they are not primary amines.

Since Leu.4 and Leu.5 have very similar chemical properties and spectroscopic characteristics, they are two diastereomers differing only at C-4 (γ-carbon): *trans*- and *cis*-4-methyl-L-proline (see the structures in Scheme 1). Identification of the two was made by comparison of the coupling constants of their α-carbon protons with those reported for the synthetic ones [23]. Leu.4 was assigned as (2*S*,4*R*)-4-methylproline (*trans*-4-methyl-L-proline), while Leu.5 was assigned as (2*S*,4*S*)-4-methylproline (*cis*-4-methyl-L-proline).

Reaction mechanisms for forming oxidation products of leucine

We have previously proposed a reaction mechanism for the generation of valine hydroperoxides by HO$^\bullet$ attack on valine [11], which involved initial formation of a carbon-centred radical as a result of hydrogen abstraction by HO$^\bullet$ and subsequent formation of a peroxyl radical species via oxygen addition. We believe that formation of leucine hydroperoxides from radiolysis of leucine follows the same reaction mechanism proposed for valine hydroperoxides. The predominant formation of γ-hydroperoxyleucine (61 μM) over that of δ-hydroperoxyleucines (31 μM) is consistent with the fact that formation of a tertiary carbon-centred radical is favoured over that of a secondary or a primary carbon-centred radical, due to stability differences between the radicals formed. The site for formation of a carbon-centred radical can also be directed by other factors [24]. For example, it has been well documented that protonated α-amino groups in amino acids de-activate the carbon sites nearby with respect to hydrogen atom abstraction by HO$^\bullet$ [25,26]. This probably explains why there is no detectable formation of β-hydroperoxyleucines during exposure of leucine to HO$^\bullet$.

The formation of the two diastereomers of 4-methyl-L-proline probably follows the reaction mechanisms proposed in Scheme 2. Hydrogen abstraction from the methyl carbon in leucine by HO$^\bullet$ radicals generates a carbon-centred radical which on reaction with oxygen forms a peroxyl radical. This peroxyl radical either gives rise to δ-hydroperoxyleucine (not shown in the Scheme) or forms an alkoxyl radical (Scheme 2) via formation of a short-lived tetroxide (results not shown). The alkoxyl radical undergoes 1,2 shift rearrangement, further addition of oxygen and elimination of a hydroperoxyl radical ($HO_2^\bullet$) to form a leucine aldehyde. The newly generated carbonyl group reacts intramolecularly with the amino group (-NH_2) to form a Schiff base, which gives rise to 4-methylproline on reduction by $NaBH_4$. The formation of valine aldehyde from radiolysis of valine, which we characterized previously [11], is analogous to the formation of the leucine aldehyde intermediate. The valine aldehyde does not form a Schiff base with its own amine group, as formation of a four-membered ring structure is not favoured compared with a five- or six-membered ring. Scheme 2 points out that 4-methylprolines are not the original oxidation products of leucine by HO$^\bullet$, but rather the reduced products of their parent imines. By HPLC fractionation of the radiolysed leucine samples before $NaBH_4$ reduction (results not shown) and re-chromatography of individual peaks after $NaBH_4$ treatment (using the same HPLC conditions described in Figure 4 for fractionation of protein hydrolysates on an analytical LC-NH_2 column), we conclude that the (non-reduced) compounds eluted at 10.7 min and 11.5 min are indeed the parent molecules, since they give rise to Leu.4 (8.9 min) and Leu.5 (9.6 min) respectively.

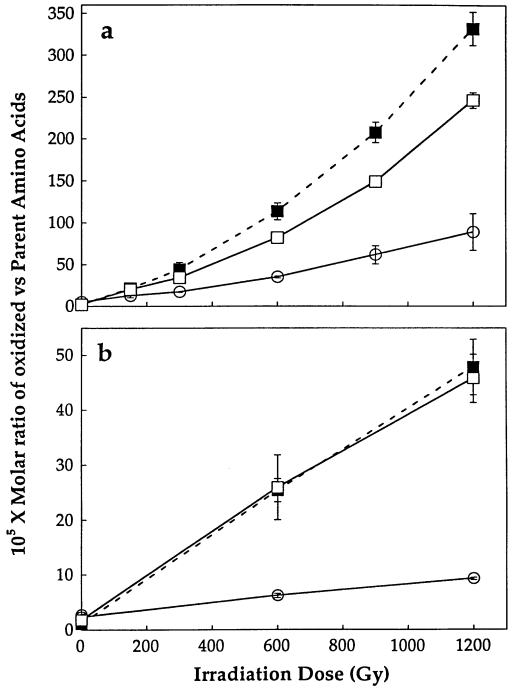


Figure 4 Detection of β-hydroxyvaline (Val.OH1) and δ-hydroxyleucines (Leu.OH2,3) on γ-radiolysed BSA (a) and human LDL (b) molecules

BSA (2 mg/ml in water) and human LDL (1 mg protein/ml in 10 mM phosphate buffer, pH 7.4) were exposed to different doses of γ-rays in the presence of oxygen. After treatment with $NaBH_4$, samples were delipidated (in the case of LDL) and hydrolysed under acid-catalysed and gas-phase conditions (see the Materials and methods section). The hydrolysate was first chromatographed on an analytical LC-NH_2 column (25 cm $\times$ 4.6 mm; 5 μm particle size; Supelco) with 81% acetonitrile in 10 mM sodium phosphate, pH 4.3, as the eluant at 1.5 ml/min, and the fraction between 12.5 and 14.6 min was collected (Val.OH1: retention time 13.2 min; Leu.OH2: 13.6; and Leu.OH3: 14.2). The collected fraction was concentrated and subjected to OPA-HPLC analysis. Data are expressed as $10^5\times$ the molar ratios Val.OH1/Val (○), $10^5\times$ Leu.OH2/Leu (■) and Leu.OH3/Leu (□). Results are means $\pm$S.D. from a single experiment ($n=3$) representative of several. If the data are expressed in units of pmol/mg of protein, the unirradiated values of Val.OH1, Leu.OH2 and Leu.OH3 would be 22.5 ± 5.0, 13.5 ± 4.0 and 14.3 ± 4.3 respectively for BSA, and 5.5 ± 1.5, 5.5 ± 0.5 and 7.0 ± 3.0 respectively for LDL. After exposure to 1200 Gy γ-irradiation, the values became 397 ± 98, 2517 ± 150 and 1868 ± 71, correspondingly, for BSA, and 22.5 ± 0.5, 240 ± 26 and 229 ± 22 for LDL.

Detection of leucine hydroxides in oxidized peptide and protein samples

^{60}Co radiolysis of Ala-Val-Leu (1 mM in water, 1200 Gy), BSA (2 mg/ml in water) and LDL (1 mg of protein/ml in 10 mM phosphate buffer, pH 7.4) was carried out in the oxygen-saturated system. As in previous experiments [8,11], significant yields of peptide-bound or protein-bound hydroperoxides were detected by iodometric assay (and were not measurable after $NaBH_4$ treatment). To avoid loss of hydroperoxides other than in the formation of hydroxides, the γ-radiolysed samples were allowed to react with $NaBH_4$ prior to complete amino acid hydrolysis. The LDL samples were de-lipidated (see the Materials and methods section) before hydrolysis. The hydrolysates were analysed using the OPA-HPLC method.

The recoveries of the hydroxyleucine residues were around 70% for δ-hydroxyleucine (Leu.OH2 and Leu.OH3) and less than 5% for γ-hydroxyleucine (Leu.OH1). In the previous study with radiolysis of valine [11] we observed that β-hydroxyvaline and γ-hydroxyvaline recoveries were 80 and 20% respectively from protein hydrolysis. The low recoveries of γ-hydroxyamino acids are probably due to their readily forming γ-lactones under acidic conditions.

The cyclic imines are not expected to be major oxidation products of proteins, since the majority of the leucine residues in proteins do not have a free amino group (-NH_2) and so are more likely to form leucine aldehydes as final products (see Scheme 2); these are reducible to δ-hydroxyleucine by $NaBH_4$. Therefore β-hydroxyvaline and δ-hydroxyleucine, either as biological reduction products of the hydroperoxides [12] or as $NaBH_4$ reduction products of the aldehydes, become candidate compounds to be examined in hydrolysates of oxidized proteins or peptides.

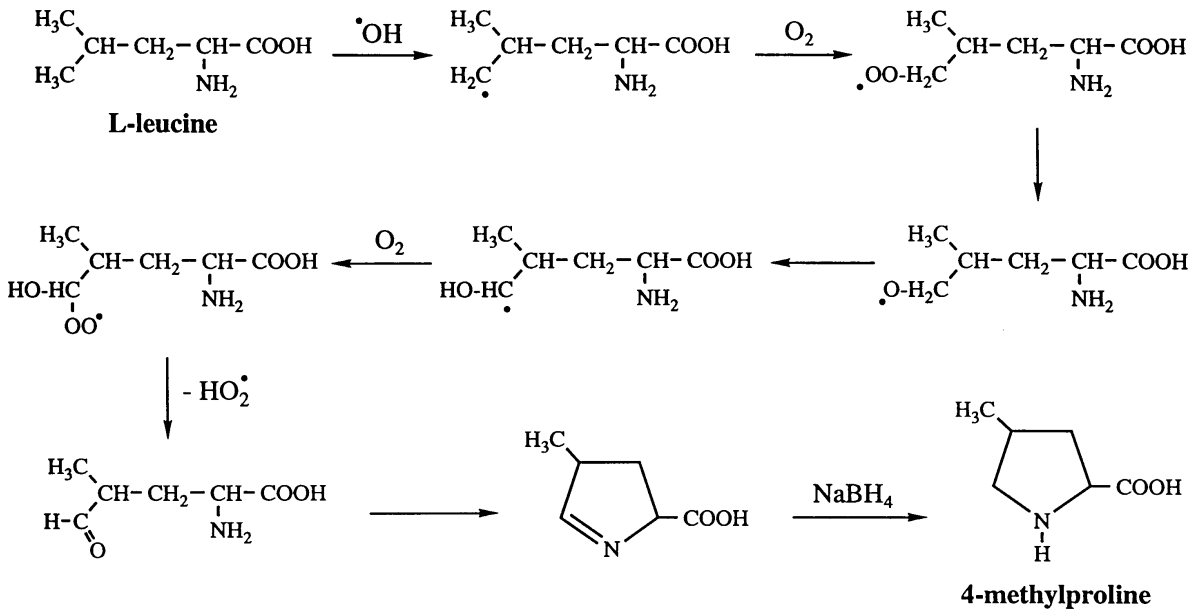
The formation of Leu.OH2 and Leu.OH3 in γ-radiolysed and $NaBH_4$-reduced Ala-Val-Leu solutions was determined to be 30 μM and 31 μM respectively, after taking into account the respective recoveries. β-Hydroxyvaline in the same sample was found to be 9 μM.

Owing to the low concentration of hydroxyleucine in oxidized proteins, its detection requires an additional purification HPLC step on an LC-NH_2 column (25 cm $\times$ 4.6 mm; 5 μm particle size; Supelco) prior to the OPA-HPLC assay. A slightly modified version of our method for detection of β-hydroxyvaline from oxidized proteins [11] was used for this purpose: the eluting window (12.5–14.6 min) of both β-hydroxyvaline and δ-hydroxyleucine was collected (with the slightly modified mobile phase of 81% acetonitrile in 10 mM sodium phosphate buffer, pH 4.3, eluted at 1.5 ml/min). This fraction was then derivatized with OPA and analysed using HPLC on an ODS column for β-hydroxyvaline and δ-hydroxyleucine. Generation of these hydroxides during irradiation of BSA (Figure 4a) and LDL (Figure 4b) showed a dose-dependent increase from 150 to 1200 Gy.

Our data revealed that the yields of oxidized amino acid per mol of parent amino acid were lower for LDL than for BSA. This is in agreement with our previous studies of lipid-associated protein [27], which showed that lipid competes with protein for incident radicals. Our data also showed that the generation of hydroxyleucine on oxidized Ala-Val-Leu, BSA and LDL was much more profound than that of hydroxyvaline (Figures 4a and 4b). In the case of BSA, the difference exceeds the difference in the number of valine and leucine residues present (36 and 61 respectively). This is in contrast with the situation with radiolysis of individual free amino acids of valine and leucine, where both hydroxyvaline and hydroxyleucine are generated comparably. There are many possible explanations. Valine residues in BSA might be less accessible than leucine to $HO^{\bullet}$, but the differential action on Ala-Val-Leu is not likely to be explained in this way. Formation of leucine aldehydes in oxidized proteins may also be a contributing factor, since they form δ-hydroxyleucine on reaction with $NaBH_4$. This is very likely, since formation of the cyclic imines from the aldehydes requires a free α amino group (see Scheme 2). The differential oxidation of valine and leucine when they are co-existing may well be due to their different reaction rates towards $HO^{\bullet}$ [14]. Last, but not least, an intermolecular radical-transfer process may be involved in the system, where valine radicals were transfered intermolecularly to leucine.

Competition between leucine and other amino acids for $HO^{\bullet}$

The saturation levels for the product generation in response to radiolysis were achieved at 5 mM for both the amino acids valine and leucine (results not shown). To understand the apparently preferential attack of $HO^{\bullet}$ on leucine, γ-radiolysis of a mixture of free leucine (10 mM) and valine (10 mM) in water was conducted in the presence of oxygen. Because radiolysed amino acids do not



Scheme 2 Proposed reaction mechanism for forming 4-methylproline during radiolysis of leucine in the presence of oxygen

Table 4 *G* values for generation of oxidized valine, leucine, and phenylalanine during γ-radiolysis in the presence of oxygen

Val-OH includes the valine hydroxides and aldehydes [11]. Leu.OX includes the leucine hydroxides and 4-methylprolines. Tyr includes *p*-, *m*-, and *o*-tyrosines determined by using an unpublished method (S.-L. Fu and R. T. Dean, unpublished work).

	$G_{Val\text{-}OH}$	$G_{Leu\text{-}OX}$	G_{Tyr}
20 mM Val	1.9	–	–
20 mM Leu	–	1.9	–
20 mM Phe	–	–	1.8
10 mM Val + 10 mM Leu	0.47	1.54	–
10 mM Phe + 10 mM Leu	–	0.45	1.29

require hydrolysis before OPA-HPLC analysis, destruction of the oxidized amino acids by hydrolysis is not encountered. Following $NaBH_4$ treatment the samples were analysed on an LC-NH_2 column monitored at 210 nm and also on an ODS column as OPA derivatives. The formation of valine and leucine hydroxides was expressed by *G* values [24] (Table 4). The *G* value for Val-OH represents the formation of the three valine hydroperoxides and the two valine aldehydes which form valine hydroxides on reduction by $NaBH_4$ [11]. The *G* value for Leu-OX represents the three leucine hydroperoxides and the two cyclic products which are converted into 4-methylprolines on reduction by $NaBH_4$. The data showed that, when the two amino acids were radiolysed separately, they each gave the same *G* value of 1.9. However, when they were radiolysed together in a 1:1 mixture, the G value for Leu.OXs was 1.54, about 3- fold higher than that for Val-OX (0.47). For comparison, Table 4 also lists the *G* value for hydroxylation products of phenylalanine on exposure to γ-radiolysis. It has been reported that the major oxidation products of phenylalanine by $HO^{\bullet}$ attack are *m*-, *o*-, and *p*-tyrosines [28]. The formation of these tyrosine isomers from radiolysis of phenylalanine was quantified using a new HPLC method developed in this laboratory (S.-L. Fu and R. T. Dean, unpublished work; details are available from S.-L. F. on request). The *G* values for formation of tyrosines, Val-OH and

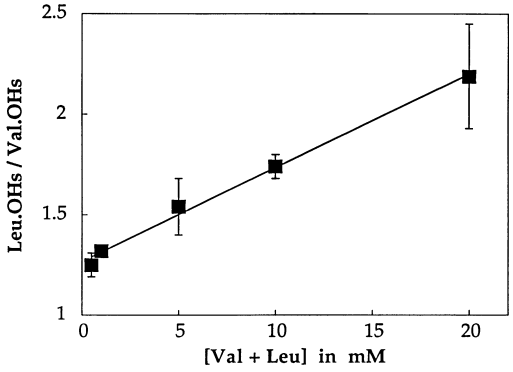


Figure 5 Generation of hydroxyleucine and hydroxyvaline from γ-radiolysis of mixture of valine and leucine (1:1) at different total concentrations

Following exposure to γ-rays (1200 Gy) in the presence of oxygen, reaction mixtures were analysed by the OPA-HPLC method (see the Materials and methods section). Data are means ± S.D. for three separate experiments.

Leu-OX are very similar (1.8 and 1.9 respectively) when the amino acids were radiolysed individually. They differed when pairs of amino acids were radiolysed together. These results are consistent with published claims that aromatic amino acids are better 'sinks' for $HO^{\bullet}$ [13,24], but also indicate that leucine has significant trapping capacity.

Do the changed *G* values in the mixtures simply reflect the difference of the reaction rates between the amino acids and $OH^{\bullet}$? If so, the ratio of *G* values would be expected to be unchanged when the total amino acid concentration is varied but their relative concentration is fixed. To address this, mixtures of valine and leucine were radiolysed at different concentrations in water while the ratio of the two was always kept at 1:1. It was found that the ratio of Leu.OHs to Val.OHs increased as the concentration of the two amino acids increased from 0.5 mM to 10 mM (Figure 5). This result suggests that generation of more oxidation products from leucine than from valine is not simply due to faster reaction of the former with $HO^{\bullet}$ [14]. In this particular case, leucine aldehydes are not contributing to the

Table 5 Detection of Leu.OH2 (δ-hydroxyleucine) in biological samples

The plasma and LDL samples were delipidated before being subjected to protein hydrolysis (see the Materials and methods section). The lens samples were powdered and hydrolysed under the same protein-hydrolysis conditions. The hydrolysates were fractionated on a LC-NH_2 analytical column (25 cm × 4.6 mm; 5 μm particle size; Supelco) with 81% acetonitrile in 10 mM sodium phosphate, pH 4.3, elution being at a rate of 1.5 ml/min. The eluting window (12.5–14.6 min) containing both β-hydroxyvaline and δ-hydroxyleucines was collected. The fraction was analysed by the OPA-HPLC method for detection of hydroxyvaline/leucine (see the Materials and methods section). The concentration of Leu.OH2 was expressed as pmol per mg of protein (or as pmol per mg of freeze-dried tissue for the lens samples) and also as molar ratio of Leu.OH2 over leucine. The values are means ± S.D. The Leu.OH3 values could not be obtained for lens samples, owing to its incomplete resolution from a contaminant peak during the OPA-HPLC analysis. From the radiolysis study of peptides, BSA and LDL (see the text and Figure 4), the generation of Leu.OH3 was found to be very comparable with that of Leu.OH2.

Biological sample	Number	[Leu.OH2] (pmol/mg of protein)	10^6 × Leu.OH2/leucine
Native human plasma	4	14.1 ± 2.9	11.0 ± 3.1
Native human LDL	3	7.1 ± 1.4	13.2 ± 2.2
Normal human lenses	3	7.8 ± 4.7	13.6 ± 8.2
Type IV cataractous human lenses	3	96.4 ± 21.6	169.7 ± 42.5

yield of hydroxyleucines, as they are not the final products in the oxidation process (Scheme 2). The simplest explanation is that there was a radical-transfer process in which valine radicals were transferred intermolecularly to leucine. Apparently, this transfer process is dependent upon the concentration of the reactants, with higher concentrations increasing the chance of collision and thus accelerating the radical transfers. Alternatively, the increased animo acid concentration may increase the amino acid 'association' in water. The reaction kinetics may be strongly determined by this association status. This issue merits further study.

Biological relevance of hydroxyleucine

There have been some previous reports of oxidized valine and leucine in biological systems. For example, in studying the structure of mutant haemoglobins, Brennan et al. [29] reported post-translational oxidation of $Leu^{\beta 141}$, and proposed hydroxyleucine as the product of a radical-mediated process. However, the authors were unable to elucidate the structure of the compound, since it was not detectable by their standard amino acid analysis. Assuming that the amino acid separation they used could resolve hydroxylated leucine from the parent and other amino acids, then, from our data, γ-hydroxyleucine is a good candidate, since it is recovered very poorly from protein hydrolysis. β-Hydroxyleucine, not detected in our study, is expected to survive reasonably well during standard hydrolysis procedures, by analogy with β-hydroxyvaline.

To address whether these *in vitro* oxidation products of protein-bound leucine by $HO^{\bullet}$ attack have any *in vivo* relevance, we have analysed several biological samples. These include native human plasma, native human LDL, native human lenses and cataractous human lenses. The plasma and LDL samples were delipidated (see the Materials and methods section) before being subjected to protein hydrolysis. The lens samples were powdered and hydrolysed under the same protein-hydrolysis conditions. The presence of hydroxyleucine in these samples was determined using the same methodology as for the γ-radiolysed BSA and LDL samples described above. The concentrations of Leu.OH2 in these biological samples are summarized in Table 5. That there are detectable hydroxyleucines in carefully isolated normal human LDL has already been documented in Figure 4(b).

The data in Table 5 indicate that hydroxyleucine is present not only in diseased tissues, but also in 'normal' fluids and tissues. Their presence at a basal level found in plasma, LDL and normal lenses may arise from dietary incorporation as well as from local oxidation. However, the more-than-10-fold difference between the normal lenses and the cataractous lenses cannot readily be accounted for by a dietary difference. *In vivo* protein oxidation is likely to be involved. We believe that the present study provides a new methodology for studying protein oxidation in biological systems under oxidative stress by using hydroxyleucine and hydroxyvaline as *in vivo* markers.

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