Biochem. J. (1991) 276, 447–453 (Printed in Great Britain)

Radical adducts of nitrosobenzene and 2-methyl-2-nitrosopropane with 12,13-epoxylinoleic acid radical, 12,13-epoxylinolenic acid radical and 14,15-epoxyarachidonic acid radical

Identification by h.p.l.c.–e.p.r. and liquid chromatography–thermospray–m.s.

Hideo IWAHASHI,* Carol E. PARKER, Ronald P. MASON and Kenneth B. TOMER

Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709, U.S.A.

Linoleic acid-derived radicals, which are formed in the reaction of linoleic acid with soybean lipoxygenase, were trapped with nitrosobenzene and the resulting radical adducts were analysed by h.p.l.c.–e.p.r. and liquid chromatography–thermospray–m.s. Three nitrosobenzene radical adducts (peaks I, II and III) were detected; these gave the following parent ion masses: 402 for peak I, 402 for peak II, and 386 for peak III. The masses of peaks I and II correspond to the linoleic acid radicals with one more oxygen atom [L(O)$^{\cdot}$]. The radicals are probably carbon-centred, because the use of $^{17}O_2$ did not result in an additional hyperfine splitting. Computer simulation of the peak I radical adduct e.p.r. spectrum also suggested that the radical is carbon-centred. The peak I radical was also detected in the reaction of 13-hydroperoxylinoleic acid with $FeSO_4$. From the above results, peak I is probably the 12,13-epoxylinoleic acid radical. An h.p.l.c.–e.p.r. experiment using [9,10,12,13-$^2H_4$]linoleic acid suggested that the 12,13-epoxylinoleic acid radical is a C-9-centred radical. Peak II is possibly an isomer of peak I. Peak III, which was observed in the reaction mixture without soybean lipoxygenase, corresponds to a linoleic acid radical (L$^{\cdot}$). The 12,13-epoxylinoleic acid radical, 12,13-epoxylinolenic acid radical and 14,15-epoxyarachidonic acid radical were also detected in the reactions of linoleic acid, linolenic acid and arachidonic acid respectively, with soybean lipoxygenase using nitrosobenzene and 2-methyl-2-nitrosopropane as spin-trapping agents.

INTRODUCTION

In the reactions of polyunsaturated fatty acids with soybean lipoxygenase, the radical intermediates have been inferred from the chemical structures of the reaction products [1]. The existence of a radical intermediates has been demonstrated in the reactions of unsaturated fatty acids with soybean lipoxygenase [2–4] and with prostaglandin synthetase [5] by e.p.r. spectroscopy. On the basis of results using ^{2}H-labelled substrates [2] de Groot *et al.* concluded that 2-methyl-2-nitrosopropanol, a spin-trapping agent, reacts mainly at position 13 and/or 9 of the linoleic acid radical.

With e.p.r. spin-trapping techniques, determination of the structures of the radical adducts has been mainly based on the hyperfine splitting pattern of the e.p.r. spectra. Hyperfine coupling constants can give detailed information about the radical centre, but no information about molecular mass. In order to obtain the comprehensive knowledge about structures of the radical adducts, additional information, such as that obtained by m.s., is necessary. Recently, we have been developing a technique for the determination of the mass spectra of radicals in which h.p.l.c.–e.p.r. and liquid chromatography–thermospray–m.s. (l.c.–TSP–m.s.) are performed under the same h.p.l.c. conditions [6]. Here we use this methodology to identify the molecular structure of nitrosobenzene and 2-methyl-2-nitrosopropane radical adducts formed by trapping linoleic acid-, linolenic acid- and arachidonic acid-derived radicals.

MATERIALS AND METHODS

Materials

Linoleic acid (octadeca-9,12-dienoic acid), linolenic acid (octadeca-9,12,15-trienoic acid), arachidonic acid (eicosa-5,8,11,14-tetraenoic acid), and 2-methyl-2-nitrosopropane were purchased from Aldrich (Milwaukee, WI, U.S.A.). [5,6,8,9,11,12,14,15-$^2H_8$]Arachidonic acid and the sodium salt of [9,10,12,13-$^2H_4$]linoleic acid were from Cambridge Isotope Laboratories (Woburn, MA, U.S.A.). Soybean lipoxygenase (type I) and nitrosobenzene were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The Sep-pak C_{18} was from Waters Associates (Milford, MA, U.S.A.). The 13-hydroperoxylinoleic acid was prepared from linoleic acid as described by Funk *et al.* [7]. The 15-hydroperoxyarachidonic acid was kindly given by Dr. W. Chamulitrat. All other chemicals used were commercial products of the highest grade available.

Reaction conditions

Unless otherwise noted, the standard reaction mixtures of nitrosobenzene and polyunsaturated fatty acids (linoleic acid, linolenic acid and arachidonic acid) consisted of 30 ml of 0.2 M-borate buffer, pH 9.0, containing 10 mM-nitrosobenzene dissolved in 1.5 ml of methanol, 6.4 mM-linoleic acid (or linolenic acid or arachidonic acid), and 13600 units of soybean lipoxygenase/ml. The reaction was started by the addition of the lipoxygenase and was allowed to proceed at 25 °C for 1 min. After 30 ml of 0.2 M-boric acid had been added, the reaction mixture was applied to the Sep-pak C_{18} column, washed with 3 ml of water, and eluted with 1 ml of acetonitrile. A 0.1 ml aliquot of the eluate was applied to an h.p.l.c.–e.p.r. set-up with a μBondapak C_{18} [300 mm (length) × 4.6 mm (internal diameter)] column to separate the spin adducts.

The reaction mixtures of 2-methyl-2-nitrosopropane and polyunsaturated fatty acids (linoleic acid and arachidonic acid)

Abbreviations used: l.c., liquid chromatography; TSP, thermospray; L$^{\cdot}$, linoleic acid radical.

* Permanent address and address for correspondence: Department of Chemistry, Wakayama Medical College, 640 Wakayama, Japan.

consisted of 5 ml of 0.2 M-borate buffer, pH 9.0, containing 23 mM-2-methyl-2-nitrosopropane, 1 mM-linoleic acid (or arachidonic acid) dissolved in 50 μl of ethanol, and 13600 units of soybean lipoxygenase/ml. The other reaction conditions were the same as for the reaction mixture of nitrosobenzene, with the exception of the reaction time, which was 3 min. The reaction mixture rapidly became anaerobic, since we used a large amount of soybean lipoxygenase (13600 units/ml) [8].

The reaction mixtures of 13-hydroperoxylinoleic acid (or 15-hydroperoxyarachidonic acid) with $FeSO_4$ (or $FeCl_3$ or soybean lipoxygenase) contained 0.5 mM-13-hydroperoxylinoleic acid (or 15-hydroperoxyarachidonic acid), 1.0 mM-$FeSO_4$ dissolved in 10 μl of 0.1 M-HCl (or 1.0 mM-$FeCl_3$ dissolved in 10 μl of 0.1 M-HCl or 13600 units of soybean lipoxygenase/ml dissolved in 10 μl of 0.2 M-borate buffer, pH 9.0), and 10 mM-nitrosobenzene dissolved in 50 μl of methanol or 23 mM-2-methyl-2-nitrosopropane in 1 ml of 0.2 M-borate buffer, pH 9.0. The reaction was started by adding $FeSO_4$ (or $FeCl_3$ or soybean lipoxygenase). The sampling procedures for h.p.l.c.–e.p.r. analyses of the reaction mixtures were the same as those for the standard reaction mixtures of the polyunsaturated fatty acids (linoleic acid, linolenic acid and arachidonic acid).

E.p.r. measurement

E.p.r. spectra were obtained using a Varian E-104 e.p.r. spectrometer of 100 kHz modulation frequency. The samples were aspirated into a Teflon tube centred in a microwave cavity. The e.p.r. spectrometer settings for 2-methyl-2-nitrosopropane (nitrosobenzene) radical adducts were: microwave power, 20 mW; modulation amplitude, 0.1 mT (0.025 mT for nitrosobenzene radical adducts); time constant, 1.0 s (0.5 s for nitrosobenzene radical adducts); scan range, 8 mT scan time, 8 min. The spectra were recorded at room temperature. E.p.r. simulations were performed using a computer program for correlation analysis of the e.p.r. spectrum [9].

H.p.l.c.–e.p.r.

H.p.l.c.–e.p.r. spectroscopy was done with an h.p.l.c. system equipped with an e.p.r. spectrometer as a detector [10–13]. H.p.l.c.–e.p.r. was performed by using a Waters model 6000A solvent-delivery system with a Varian E-104 e.p.r. spectrometer. The e.p.r. spectrometer was connected to the h.p.l.c. system with a Teflon tube that passed through the e.p.r. tube cavity. The magnetic field of the e.p.r. spectrometer was fixed at the low-field peak (nitrosobenzene) and at the middle peak (2-methyl-2-nitrosopropane) of the e.p.r. spectra. The e.p.r. settings were: microwave power, 20 mW; modulation amplitude, 0.8 mT; modulation frequency, 100 kHz; time constant, 1 s. H.p.l.c. column conditions were as follows: flow rate, 1.0 ml/min; injection volume, 0.1 ml; gradient elution [solvent A, 10 mM-ammonium acetate/20% (v/v) acetonitrile; solvent B, 10 mM-ammonium acetate/80% (v/v) acetonitrile] from 20% B to 70% B in 30 min.

L.c.–TSP–m.s.

The mass spectrometer used for these experiments was a VG 12-250 quadrupole mass spectrometer/data system (VG Masslab, Altrincham, Cheshire, U.K.) equipped with a Vestec model 701S source (Vestec Corp., Houston, TX, U.S.A.). The mass range scanned was from 125 to 800 Da at 2 s/scan in the negative-ion mode. Typical operating conditions for the Vestec TSP source were: control temperature (T_1), 80 °C; tip temperature, 235 °C; vapour temperature, 221 °C; block temperature, 290 °C; tip heater, 245 °C; lens, 30 °C. The repeller was set for negative ions at 0 V and the discharge electrode was on at 200 μA. The h.p.l.c. conditions for l.c.–TSP–m.s. analysis were the same as for h.p.l.c.–e.p.r. [6].

RESULTS

E.p.r. spectra of the radical adducts of nitrosobenzene with linoleic acid-derived radicals

E.p.r. measurements on the reaction mixture of linoleic acid with soybean lipoxygenase were performed with nitrosobenzene as the spin-trapping agent (results not shown). The e.p.r. spectrum is similar to the one reported by Aoshima *et al.* [3]. A similar e.p.r. signal, but of reduced intensity, was also observed in the reaction mixture without soybean lipoxygenase (results not shown), suggesting the generation of both lipoxygenase-dependent and lipoxygenase-independent radicals. The reaction mixture without linoleic acid gave no e.p.r. signal.

H.p.l.c.–e.p.r. analysis of the reaction mixture of linoleic acid, soybean lipoxygenase and nitrosobenzene

H.p.l.c.–e.p.r. analysis was performed on the reaction mixture of linoleic acid, soybean lipoxygenase and nitrosobenzene. Fig 1(*a*) shows the u.v. trace at 280 nm of the h.p.l.c. analysis of the complete reaction mixture. Three peaks (I, II and III) were observed in the h.p.l.c.–e.p.r. elution pattern of the complete reaction mixture at retention times of 29.0, 30.0 and 35.6 min respectively (Fig. 1*b*). Peak III was also detected for the reaction mixture without soybean lipoxygenase (Fig. 1*c*), suggesting that the peak III radical adduct is formed non-enzymically. These non-

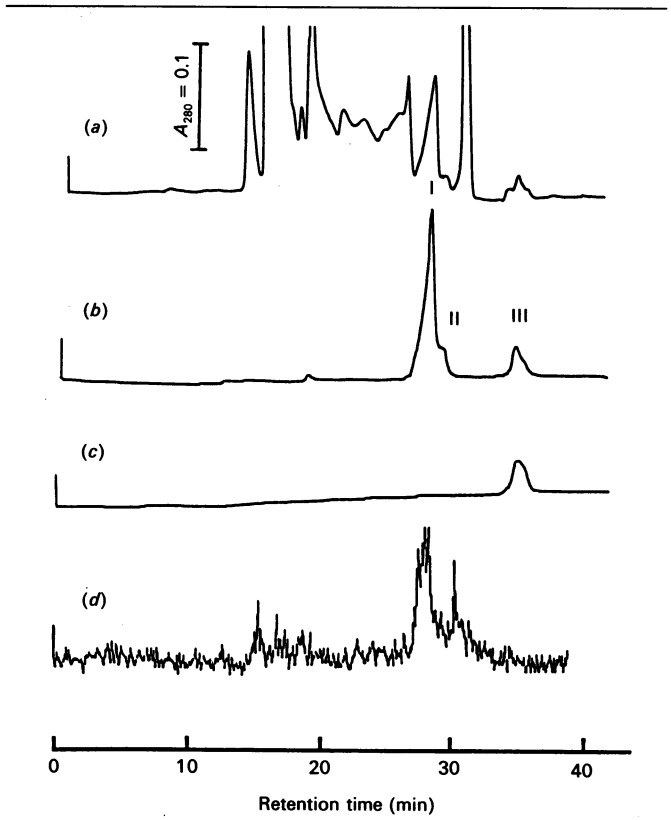


Fig. 1. H.p.l.c.–e.p.r. and l.c.–TSP–m.s. analyses of the reaction mixture containing linoleic acid, soybean lipoxygenase and nitrosobenzene

Conditions for the reaction and analyses (h.p.l.c.–e.p.r. and l.c.–TSP–m.s.) were as described in the Materials and methods section. (*a*) U.v. trace (280 nm) of the h.p.l.c. analysis of the complete reaction mixture. (*b*) H.p.l.c.–e.p.r. analysis of the complete reaction mixture. The receiver gain of the e.p.r. spectrometer was 1.25×10^4. (*c*) H.p.l.c.–e.p.r. analysis of the reaction mixture without soybean lipoxygenase. The receiver gain of the e.p.r. spectrometer was 1.25×10^4. (*d*) L.c.–TSP–m.s. analysis of the complete reaction mixture. A reconstructed mass chromatogram for m/z 402 is shown for the complete reaction mixture.

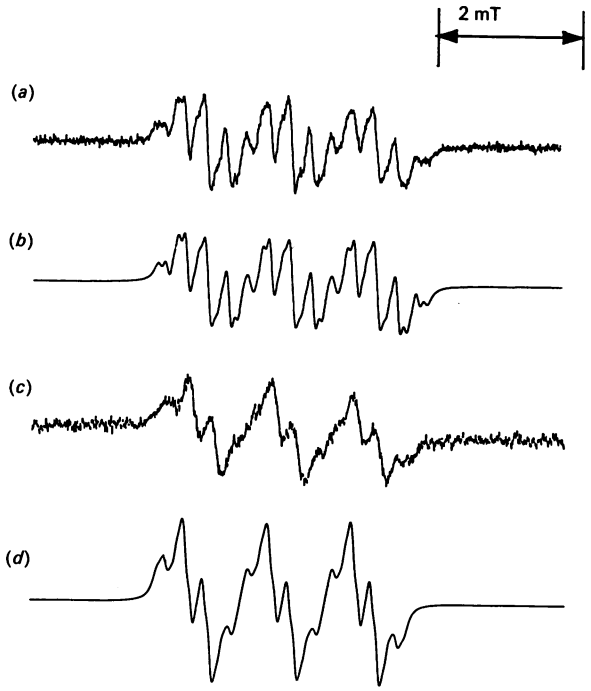


Fig. 2. E.p.r. spectrum of peak I in the h.p.l.c.–e.p.r. analysis for the reaction mixture of linoleic acid, soybean lipoxygenase and nitrosobenzene

(*a*) E.p.r. spectrum of peak I. The peak I radical adduct was dissolved in 10 mM-ammonium acetate/70% (v/v) acetonitrile. (*b*) Simulated spectrum of (*a*). Hyperfine coupling constants used here are as follows: $a^N = 1.17$ mT, $a_{o,p}^H = 0.27$ mT, $a_m^H = 0.1$ mT, $a_\beta^H = 0.31$ mT. (*c*) E.p.r. spectrum of peak I when [9,10,12,13-$^2H_4$]linoleic acid was used. The peak I radical adduct was dissolved in 10 mM-ammonium acetate/70% (v/v) acetonitrile. (*d*) Simulated spectrum of (*c*). Hyperfine coupling constants used here are as follows: $a^N = 1.17$ mT, $a_{o,p}^H = 0.27$ mT, $a_m^H = 0.1$ mT, $a_\beta^H = 0.05$ mT.

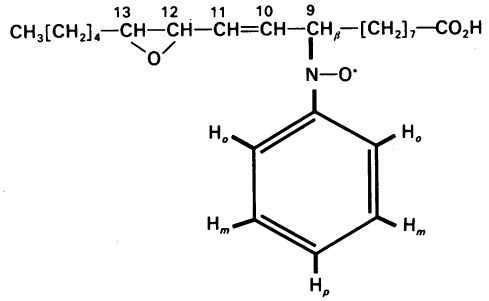


Fig. 3. Proposed structure of the nitrosobenzene radical adduct formed by trapping a linoleic acid-derived radical

enzymic reactions might occur through a pseudo-Diels–Alder mechanism (or an 'ene' reaction) [14,15]. Peaks I, II, and III were also observed in the u.v. trace at 280 nm of the h.p.l.c. elution pattern. Peak I was collected and its e.p.r. spectrum was measured in 10 mM-ammonium acetate/70% (v/v) acetonitrile (Fig. 2*a*). Computer simulation of the peak I radical adducts gave the following hyperfine coupling constants: $a^N = 1.17$ mT, $a_{o,p}^H = 0.27$ mT, $a_m^H = 0.1$ mT, $a_\beta^H = 0.31$ mT (Fig. 2*b*). Taking into account the relatively large hyperfine coupling constant ($a_\beta^H = 0.31$ mT) due to the β-proton of the radical, the radical is probably carbon-centred (Fig. 3).

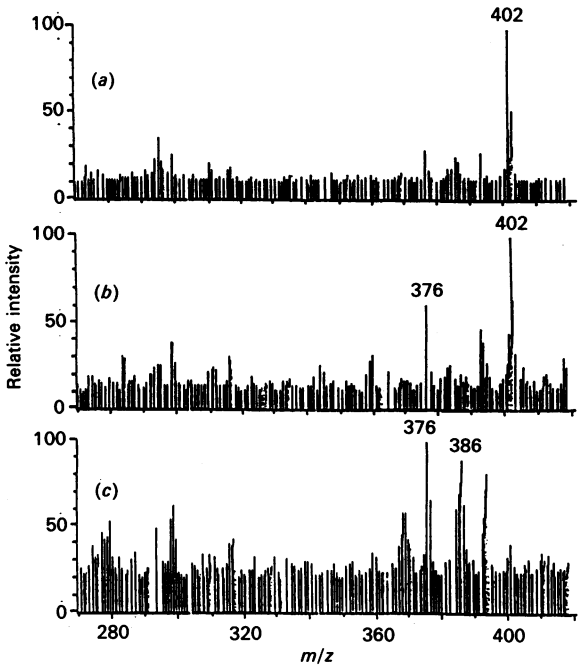


Fig. 4. Mass spectra of peaks I, II and III from h.p.l.c.–e.p.r. analysis of the reaction mixture of linoleic acid, soybean lipoxygenase and nitrosobenzene

Conditions for the reaction and l.c.–TSP–m.s. were as described in the Materials and methods section. (*a*) Peak I; (*b*) peak II; (*c*) peak III.

In order to confirm that the trapped radicals are not oxygen-centred, an experiment using $^{17}O_2$ was performed. No additional e.p.r. hyperfine splitting was detectable in the reaction mixture bubbled with $^{17}O_2$, suggesting that oxygen-centred radicals were not detected under our reaction conditions.

To obtain structural information about the trapped free-radical centres, we measured the e.p.r. spectrum of peak I with [9,10,12,13-$^2H_4$]linoleic acid in 10 mM-ammonium acetate/70% (v/v) acetonitrile. The spectrum changed dramatically from the non-^{2}H-labelled-linoleic acid spectrum (Fig. 2*c*). A good simulation of the spectrum was obtained using the same hyperfine constants as for non-^{2}H-labelled linoleic acid, except that we used 0.05 mT (0.5 G) (instead of '$a_\beta^{^2H}$' of non-^{2}H-labelled linoleic acid/6.5) for β-proton hyperfine coupling constant (Fig. 2*d*). This suggests that the β-proton is replaced by ^{2}H in the ^{2}H-labelled linoleic acid.

L.c.–TSP–m.s. analysis of the reaction mixture of linoleic acid, soybean lipoxygenase and nitrosobenzene

L.c.–TSP–m.s. analysis was performed on the reaction mixture of linoleic acid, soybean lipoxygenase and nitrosobenzene. The reconstructed l.c.–TSP–m.s. chromatogram for *m/z* 402 showed peaks corresponding to peaks I and II of the h.p.l.c.–e.p.r. trace (Fig. 1*d*). The reconstructed l.c.–TSP–m.s. chromatogram for *m/z* 386 gave a peak corresponding to peak III (results not shown). Mass spectra of the peaks (I, II and III) are shown in Fig. 4. The ion at *m/z* 376 in the mass spectra is due to background.

H.p.l.c.–e.p.r. analysis of the reaction mixture of 13-hydroperoxylinoleic acid, $FeSO_4$ and nitrosobenzene

In order to determine whether the peak I radical adduct is formed from 13-hydroperoxylinoleic acid or not [16,17],

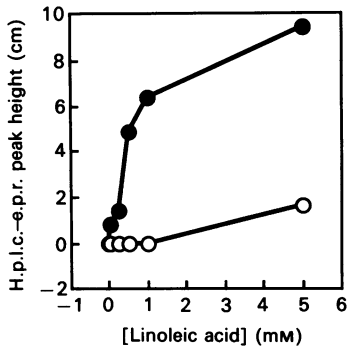


Fig. 5. Linoleic acid-concentration-dependence of the h.p.l.c.–e.p.r. peak height of peaks I and III

The reaction systems contained, in 1 ml of 0.2 M-borate buffer, pH 9.0, 10 mM-nitrosobenzene, indicated concentrations of linoleic acid dissolved in 50 μl of ethanol, and 1.36×10^4 units of soybean lipoxygenase/ml. The other reaction and h.p.l.c.–e.p.r. conditions were as described in the Materials and methods section. ●, Peak I; ○, peak III.

Table 1. H.p.l.c.-e.p.r. peak height of peak I in different reaction systems

The reaction systems contained [in 1 ml of 0.2 M-borate buffer (pH 9.0)], 10 mM-nitrosobenzene, 500 μM-linoleic acid (or 500 μM-13-hydroperoxylinoleic acid) and 13600 units of soybean lipoxygenase/ml (or 1 mM-$FeSO_4$ or 1 mM-$FeCl_3$). The other reaction and h.p.l.c.–e.p.r. analysis conditions were as described in the Materials and methods section.

System	Peak height (cm)	(%)
Soybean lipoxygenase + linoleic acid	7.3 ± 1.5	100
Soybean lipoxygenase + 13-hydroperoxy-linoleic acid	0	0
$FeSO_4$ + 13-hydroperoxylinoleic acid	6.2 ± 0.8	85
$FeCl_3$ + 13-hydroperoxylinoleic acid	1.5 ± 0.2	21

h.p.l.c.–e.p.r. analysis of the reaction mixture of 13-hydroperoxylinoleic acid with $FeSO_4$ was performed using nitrosobenzene as the spin-trapping agent (results not shown). A peak was detected at the same retention time as peak I in the h.p.l.c.–e.p.r. elution pattern of the linoleic acid reaction mixture, suggesting that peak I is a 13-hydroperoxylinoleic acid-derived radical. All the above e.p.r., h.p.l.c.–e.p.r. and l.c.–TSP–m.s. results indicate that peak I is a 12,13-epoxylinoleic acid radical adduct and peak III is a linoleic acid radical adduct (nitrosobenzene/L$^{\cdot}$). The data for peak II is consistent with it being an isomer of the 12,13-epoxylinoleic acid radical adduct.

Linoleic acid-concentration-dependence of the h.p.l.c.–e.p.r. peak height of peaks I and III

The linoleic acid-concentration-dependencies of peaks I and III were determined (Fig. 5) using nitrosobenzene as the spin-trapping agent. The 12,13-epoxylinoleic acid radical adduct (peak I) was formed at relatively low linoleic acid concentrations (0.25–1 mM). Over this concentration range the linoleic acid radical adduct (peak III) was not observed. Peak III was detected only when the concentration of linoleic acid was raised to 5 mM. The different linoleic acid-concentration-dependencies of the two peaks are consistent with the hypothesis that the adduct of nitrosobenzene with 12,13-epoxylinoleic acid radical adduct is formed enzymically, whereas the adduct of nitrosobenzene with the linoleic acid radical is formed non-enzymically.

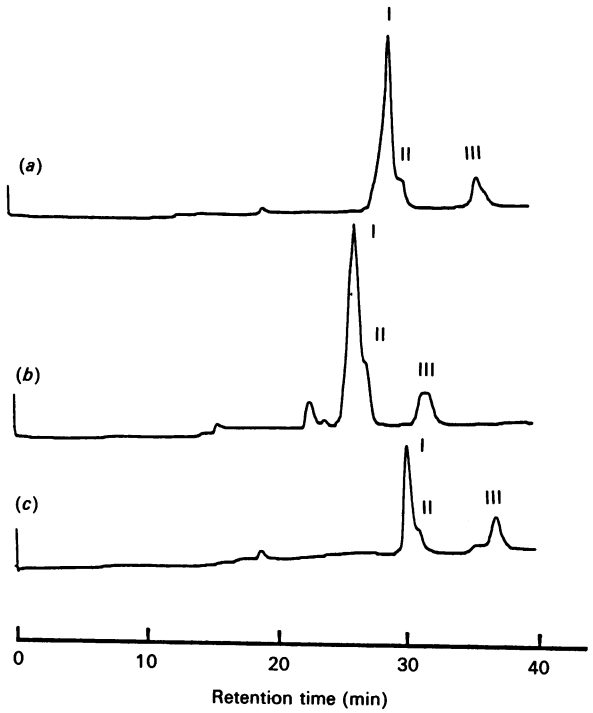


Fig. 6. H.p.l.c.–e.p.r. analysis of the nitrosobenzene radical adducts of linoleic acid-, linolenic acid- and arachidonic acid-derived radicals

The reaction mixture consisted of linoleic acid (or linolenic acid or arachidonic acid), soybean lipoxygenase and nitrosobenzene. Reaction and h.p.l.c. conditions were as described in the Materials and methods section. The receiver gain of the e.p.r. spectrometer was 1.25×10^4. (*a*) Linoleic acid; (*b*) linolenic acid; (*c*) arachidonic acid.

Nitrosobenzene radical adduct with 12,13-epoxylinoleic acid radical: formation under different reaction systems

The relative abundance of the nitrosobenzene–12,13-epoxylinoleic acid radical adduct (peak I) was measured in the reaction mixture of 13-hydroperoxylinoleic acid under different reaction conditions (Table 1). Formation of the 12,13-epoxylinoleic acid radical was observed in the reaction mixture of 13-hydroperoxylinoleic acid with $FeSO_4$. A peak of low abundance was observed in the reaction of 13-hydroperoxylinoleic acid with $FeCl_3$, and peak I was not detectable in the reaction of 13-hydroperoxylinoleic acid with soybean lipoxygenase.

H.p.l.c.–e.p.r. and l.c.–TSP–m.s. analysis of the reaction mixtures of linolenic acid (or arachidonic acid), soybean lipoxygenase and nitrosobenzene

H.p.l.c.–e.p.r. and l.c.–TSP–m.s. analyses of the reaction mixtures of linolenic acid (or arachidonic acid), soybean lipoxygenase and nitrosobenzene were performed. The h.p.l.c.–e.p.r. analysis of linolenic acid (or arachidonic acid) showed elution patterns similar to those of linoleic acid (Fig. 6). In the h.p.l.c.–e.p.r. analyses of linolenic acid and arachidonic acid, the three major peaks are at 26.0 min (I), 27.0 min (II), and 31.8 min (III) for linolenic acid, and 30.2 min (I), 30.8 min (II), and 36.9 min (III) for arachidonic acid respectively. Relative retention times of the main nitrosobenzene radical adducts of linoleic acid, linolenic acid, and arachidonic acid are identical with the results reported by Sugata *et al.* [13]. Peaks III of

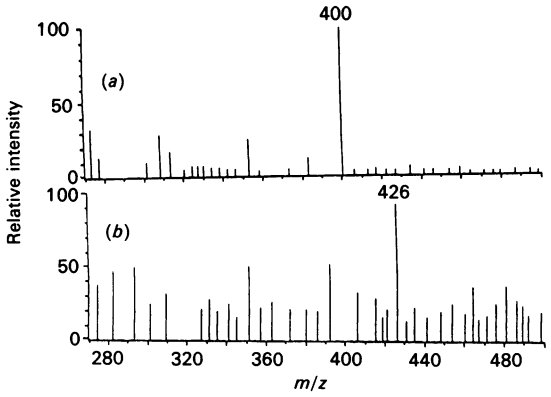


Fig. 7. Mass spectra of the nitrosobenzene radical adducts of linolenic acid- and arachidonic acid-derived radicals

The reaction mixture consisted of linolenic acid (or arachidonic acid), soybean lipoxygenase and nitrosobenzene. Reaction and l.c.–TSP–m.s. conditions were described in the Materials and methods section. (*a*) Mass spectrum of the nitrosobenzene radical adduct of 12,13-epoxylinolenic acid; (*b*) mass spectrum of the nitrosobenzene radical adduct of 14,15-epoxyarachidonic acid radical.

linolenic acid and arachidonic acid were also detected in the respective reaction mixtures without soybean lipoxygenase. The l.c.–TSP–m.s. spectra of peak I from linolenic acid and arachidonic acid showed parent ions of m/z 400 for linolenic acid and m/z 426 for arachidonic acid (Fig. 7). The reaction mixture of 13-hydroperoxylinoleic acid (15-hydroperoxyarachidonic acid), Fe^{2+} and nitrosobenzene also gave a peak corresponding to peak I of linoleic acid (or arachidonic acid) (results not shown) respectively. These data indicate the formation of 12,13-epoxylinolenic acid radical adduct and 14,15-epoxyarachidonic acid radical adduct respectively. The reconstructed l.c.–TSP–m.s. chromatograms for m/z 384 and m/z 410 gave peaks with the same retention times as those of peak III of linolenic acid and arachidonic acid in h.p.l.c.–e.p.r. respectively, suggesting that peaks III of linolenic acid and arachidonic acid are linolenic acid radical and arachidonic acid radical adducts (L$^{\cdot}$) respectively (results not shown).

Detection of the 12,13-epoxylinoleic acid radical adduct and the 14,15-epoxyarachidonic acid adducts using 2-methyl-2-nitrosopropane as the spin-trapping reagent

H.p.l.c.–e.p.r. and l.c.–TSP–m.s. analyses were performed on the reaction mixtures of linoleic acid (or arachidonic acid), soybean lipoxygenase and 2-methyl-2-nitrosopropane as spin-trapping agent. The h.p.l.c.–e.p.r. analyses of the reaction mixtures gave prominent peaks with retention times of 25.4 min (linoleic acid) (Fig. 8*a*) and 28.3 min (arachidonic acid) (Fig. 8*b*) respectively. The e.p.r. spectra of the peaks of linoleic acid-derived radical (Fig. 8*c*) and arachidonic acid-derived radical adducts (results not shown) with 2-methyl-2-nitrosopropane were measured in 10 mM-ammonium acetate and 55% (v/v) acetonitrile. Both e.p.r. spectra consisted of six lines with hyperfine coupling constants of $a^N = 1.58$ mT and $a_\beta^H = 0.22$ mT, indicative of a hydrogen atom at the β-position. H.p.l.c.–e.p.r. analyses were performed for the reaction mixtures of 13-hydroperoxylinoleic acid (or 15-hydroperoxyarachidonic acid), Fe^{2+} and 2-methyl-2-nitrosopropane. The h.p.l.c.–e.p.r. analyses gave peaks with the same retention times as those from linoleic acid and arachidonic acid respectively (results not shown).

H.p.l.c.–e.p.r. analyses were performed on reaction mixtures

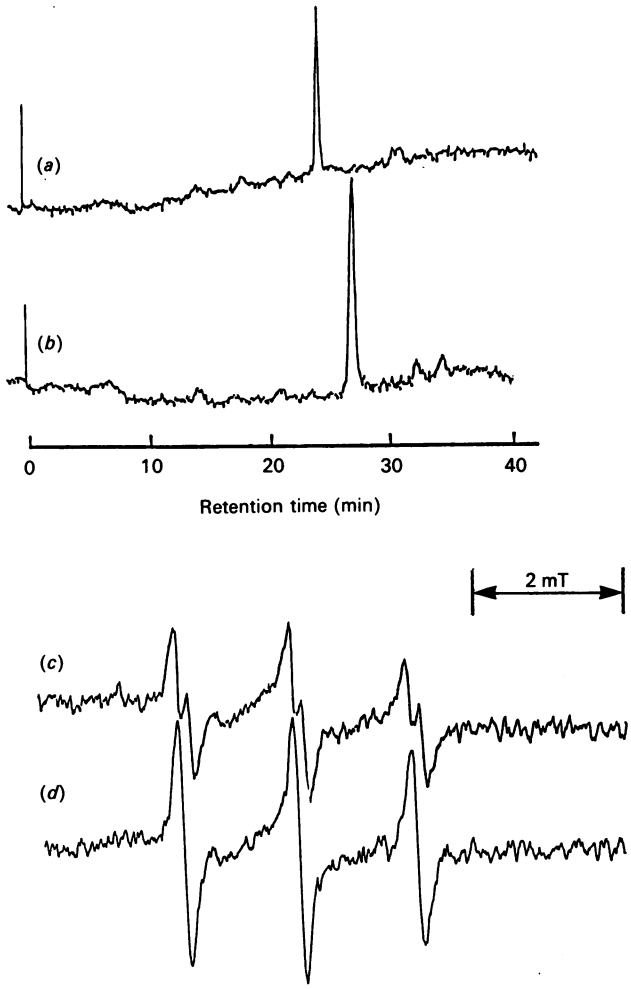


Fig. 8. H.p.l.c.–e.p.r. and e.p.r. analyses of the reaction mixtures of linoleic acid (or arachidonic acid), soybean lipoxygenase and 2-methyl-2-nitrosopropane

Conditions for the reaction and analyses (h.p.l.c.–e.p.r. and e.p.r.) were as described in the Materials and methods section. (*a*) H.p.l.c.–e.p.r. analysis of the reaction mixture of linoleic acid, soybean lipoxygenase and 2-methyl-2-nitrosopropane. The receiver gain of the e.p.r. spectrometer was 2.5×10^4. (*b*) Same as (*a*), except that arachidonic acid was used instead of linoleic acid. (*c*) E.p.r. spectrum of the h.p.l.c.–e.p.r. peak in (*a*). The receiver gain of the e.p.r. spectrometer was 5.0×10^4. The radical adduct was dissolved in 10 mM-ammonium acetate/55% (v/v) acetonitrile. (*d*) Same as (*c*), except that [9,10,12,13-$^2H_4$]linoleic acid was used instead of linoleic acid.

containing [9,10,12,13-$^2H_4$]linoleic acid (or [5,6,8,9,11,12,14,15-$^2H_8$]arachidonic acid), soybean lipoxygenase and 2-methyl-2-nitrosopropane. The hyperfine splitting due to the β-proton disappeared in the e.p.r. spectra of the h.p.l.c.–e.p.r. peaks of [^{2}H]linoleic acid (Fig. 8*d*) and arachidonic acid (results not shown), demonstrating that the β-protons are replaced by ^{2}H in the [^{2}H]linoleic acid and [^{2}H]arachidonic acid.

The l.c.–TSP–m.s. analyses of the reaction mixture containing linoleic acid (or arachidonic acid), soybean lipoxygenase and 2-methyl-2-nitrosopropane gave parent ions of m/z 381 for the linoleic acid-derived radical adduct and m/z 405 for the arachidonic acid-derived radical adduct (Fig. 9). These correspond to the masses of the radical adducts of 2-methyl-2-nitrosopropane with the 12,13-epoxylinoleic acid radical and with the 14,15-epoxyarachidonic acid radical respectively.

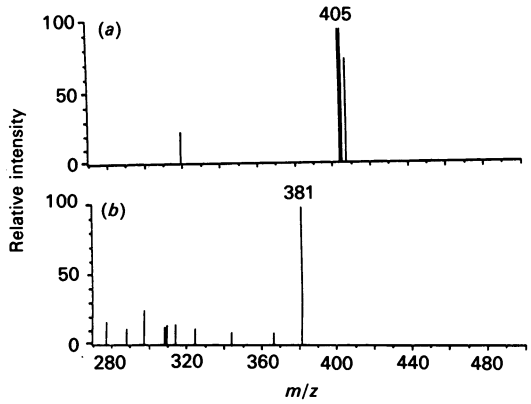
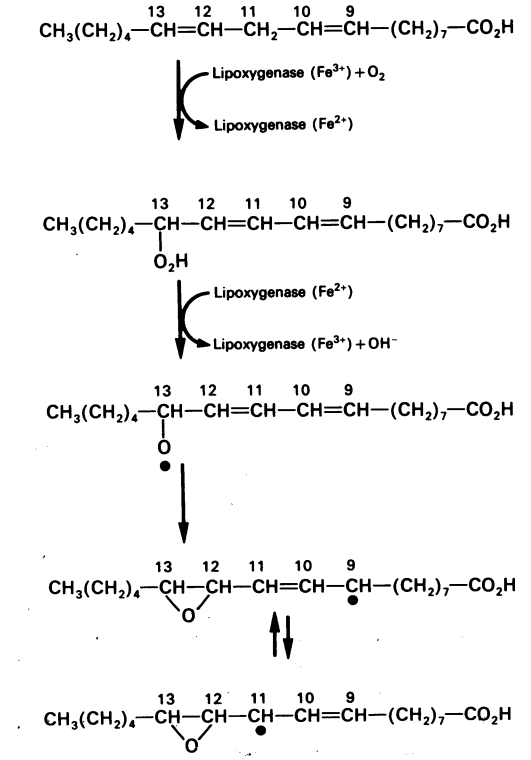


Fig. 9. Mass spectra of the 2-methyl-2-nitrosopropane radical adducts with 12,13-epoxylinoleic acid radical and 14,15-epoxyarachidonic acid radical

The reaction mixture consists of linoleic acid (or arachidonic acid), soybean lipoxygenase and 2-methyl-2-nitrosopropane. Conditions for the reaction and l.c.–TSP–m.s. analysis are as described in the Materials and methods section. (*a*) 14,15-Epoxyarachidonic acid radical. (*b*) 12,13-epoxylinolenic acid radical.

DISCUSSION

In the present study we detected three radical adducts (peaks I, II and III) of nitrosobenzene with linoleic acid-derived radicals in a reaction mixture containing linoleic acid, soybean lipoxygenase and nitrosobenzene. Peak III radical adduct was formed non-enzymically, presumably via an 'ene' reaction, as has been described [14,15]. The peak III radical corresponds to the radical adduct of nitrosobenzene with linoleic acid radical (L$^{\bullet}$). This type of radical adduct was also detected in the reaction mixtures containing either linolenic acid or arachidonic acid. The formation of peaks I and II was catalysed by soybean lipoxygenase. The molecular mass of these two peaks (I and II) corresponds to LO$^{\bullet}$. However, $^{17}O_2$ experimental results and computer simulation of the e.p.r. signal excluded the possibility of the trapping of oxygen-centred radicals. From these data we conclude that the trapped radical is the 12,13-epoxylinoleic acid radical.



Scheme 1. A possible scheme for the formation of the 12,13-epoxylinoleic acid radical

We propose a scheme to account for the formation of the 12,13-epoxylinoleic acid radical [18] (Scheme 1). First, soybean lipoxygenase catalyses the formation of 13-hydroperoxylinoleic acid. During the reaction, the iron ions in soybean lipoxygenase are reduced to the ferrous state. The 13-hydroperoxylinoleic acid is then broken down to form an alkoxy radical by soybean lipoxygenase in the ferrous state. Indeed, soybean lipoxygenase, in which the iron ions are in the ferric state, does not catalyse the formation of the 12,13-epoxylinoleic acid radical (Table 1). The alkoxy radical then isomerizes to form the 12,13-epoxylinoleic acid radical which can be trapped at the C-9 or C-11 position by nitrosobenzene or 2-methyl-2-nitrosopropane.

As described above, there are two possible resonance structures (C-9 and C-11) for the 12,13-epoxylinoleic acid radical (Scheme 1). The h.p.l.c.–e.p.r. experiment using [9,10,12,13-$^2H_4$]linoleic acid indicated that the proton of the radical centre is replaced by ^2H. Therefore the 12,13-epoxylinoleic acid radical trapped in the present study is the radical centred at position C-9. Similarly, the 14,15-epoxyarachidonic acid radical is a C-11 carbon-centred radical. On the basis of the results obtained with spin trapping of the [^2H]linoleic acid [2] de Groot *et al.* also concluded that the spin-trapping agent 2-methyl-2-nitrosopropanol reacts mainly at position 13 and/or 9 of the linoleic acid radical.

Linoleic acid radical (L$^{\bullet}$) might be formed during the peroxidation step to form 13-hydroperoxylinoleic acid. However, L$^{\bullet}$ was not detected by nitrosobenzene and 2-methyl-2-nitrosopropane in our reaction system. There are at least three possible explanations for the above results. First, the 13-hydroperoxidation of linoleic acid occurs in the active site of soybean lipoxygenase. Nitrosobenzene and 2-methyl-2-nitrosopropane might not trap the L$^{\bullet}$ radical because they cannot enter the active site of the enzyme. Secondly, the organo-iron-mediated pathway for hydroperoxidation of fatty acids by soybean lipoxygenase may explain the above results, because L$^{\bullet}$ could not be formed [19,20]. Thirdly, the nitrosobenzene and 2-methyl-2-nitrosopropane radical adducts with L$^{\bullet}$ are too unstable to be detected by h.p.l.c.–e.p.r.

Formation of the 12,13-epoxy-linoleic acid radical adduct detected here can explain the formation of various kinds of linoleic acid-related compounds such as linoleic acid dimers, which contain epoxide, from the reaction mixture of linoleic acid and its hydroperoxide with soybean lipoxygenase [1]. Products found include methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*cis*-12,13-epoxy-*trans*-octadec-9-enoate and methyl 11-(2,2,5,7,8-pentamethyl-6-oxychroman)-*trans*-12,13-epoxy-*trans*-9-octadecenoate from the reaction mixture of methyl linoleate hydroperoxide and $FeCl_3$ [21], 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid, 9-oxo-*cis*-12,13-epoxy-*trans*-10-octadecenoic acid, 11-hydroxy-*trans*-12,13-epoxy-*cis*-9-octadecadienoic acid, and 11-hydroxy-*trans*-12,13-epoxy-*trans*-9-octadecadienoic acid from the reaction mixtures of 13-hydroperoxylinoleic acid with $FeCl_3$–cysteine [22], haemoglobin [23], and soybean lipoxygenase [24] respectively, and *trans*-12,13-epoxy-9-hydroperoxy-*trans*-10-octadecenoic acid from the reaction mixture of 13-hydroperoxylinoleic acid with cysteine–$FeCl_3$ [25] or haematin [26].

In the present investigation, 2-methyl-2-nitrosopropane also trapped the 12,13-epoxylinoleic acid radical and the 14,15-epoxyarachidonic acid radical under our reaction conditions. The e.p.r. hyperfine coupling constants of the epoxy radicals are very close to the values which were reported for radical adducts

of 2-methyl-2-nitrosopropane (or 2-methyl-2-nitrosopropanol) with the linoleic acid radical and arachidonic acid radical (L$^{\bullet}$) in enzymic reaction systems [2,3,5,27,28] and other oxidation systems [29–37]. Other information, such as mass spectra and h.p.l.c. retention times, will be necessary to distinguish the epoxy radicals from the L$^{\bullet}$ species.

We thank Dr. W. Chamulitrat for generously providing the 15-hydroperoxyarachidonic acid. We also thank Mr. D. Duling for performing the computer simulations of the e.p.r. spectra.

REFERENCES

1. Garssen, G. J., Vliegenthart, J. F. G. & Boldingh, J. (1972) Biochem. J. **130**, 435–442
2. de Groot, J. J. M. C., Garssen, G. J., Vliegenthart, J. F. G. & Boldingh, J. (1973) Biochim. Biophys. Acta **326**, 279–284
3. Aoshima, H., Kajiwara, T., Hatanaka, A. & Hatano, H. (1977) J. Biochem. (Tokyo) **82**, 1559–1565
4. Chamulitrat, W. & Mason, R. P. (1989) J. Biol. Chem. **264**, 20968–20973
5. Mason, R. P., Kalyaraman, B., Tainer, B. E. & Eling, T. E. (1980) J. Biol. Chem. **255**, 5019–5022
6. Iwahashi, H., Parker, C. E., Mason, R. P. & Tomer, K. B. (1990) Rapid Commun. Mass Spectrom. **4**, 352–354
7. Funk, M. O., Isaac, R. & Porter, N. A. (1976) Lipids **11**, 113–117
8. Connor, H. D., Fischer, V. & Mason, R. P. (1986) Biochem. Biophys. Res. Commun. **141**, 614–621
9. Motten, A. & Schreiber, J. (1986) J. Magn. Reson. **67**, 42–54
10. Makino, K. & Hatano, H. (1979) Chem. Lett. 119–122
11. Makino, K., Moriya, F. & Hatano, H. (1985) J. Chromatogr. **332**, 71–106
12. Iwahashi, H., Ikeda, A., Negoro, Y. & Kido, R. (1986) Biochem. J. **236**, 509–514
13. Sugata, R., Iwahashi, H., Ishii, T. & Kido, R. (1989) J. Chromatogr. **487**, 9–16
14. Sullivan, A. B. (1966) J. Org. Chem. **31**, 2811–2817
15. Floyd, R. A., Soong, L. M., Stuart, M. A. & Reigh, D. L. (1978) Arch. Biochem. Biophys. **185**, 450–457
16. Schreiber, J., Mason, R. P. & Eling, T. E. (1986) Arch. Biochem. Biophys. **251**, 17–24
17. Hughes, M. F., Chamulitrat, W., Mason, R. P. & Eling, T. E. (1989) Carcinogenesis **10**, 2075–2080
18. de Groot, J. J. M. C., Veldink, G. A., Vliegenthart, J. F. G., Boldingh, J., Wever, R. & van Gelder, B. F. (1975) Biochim. Biophys. Acta **377**, 71–79
19. Corey, E. J. & Nagata, R. (1987) J. Am. Chem. Soc. **109**, 8107–8108
20. Corey, E. J. & Walker, J. C. (1987) J. Am. Chem. Soc. **109**, 8108–8109
21. Gardner, H. W., Eskins, K., Grams, G. W. & Inglett, G. E. (1972) Lipids **7**, 324–334
22. Gardner, H. W., Kleiman, R. & Weisleder, D. (1974) Lipids **9**, 696–706
23. Hamberg, M. (1974) Lipids **10**, 87–92
24. Garssen, G. J., Veldink, G. A., Vliegenthart, J. F. G. & Boldingh, J. (1976) Eur. J. Biochem. **62**, 33–36
25. Gardner, H. W., Weisleder, D. & Kleiman, R. (1978) Lipids **13**, 246–252
26. Dix, T. A. & Marnett, L. J. (1985) J. Biol. Chem. **260**, 5351–5357
27. Rosen, G. M. & Rauckman, E. J. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 7346–7349
28. Sekiya, J., Aoshima, H., Kajiwara, T., Togo, T. & Hatanaka, A. (1977) Agric. Biol. Chem. **41**, 827–832
29. Kalyanaraman, B., Mason, R. P., Perez-Reyes, E., Chignell, C. F., Wolf, C. R. & Philpot, R. M. (1979) Biochem. Biophys. Res. Commun. **89**, 1065–1072
30. Pryor, W. A., Prier, D. G. & Church, D. F. (1981) Environ. Res. **24**, 42–52
31. Albano, E., Lott, K. A. K., Slater, T. F., Stier, A., Symons, M. C. R. & Tomasi, A. (1982) Biochem. J. **204**, 593–603
32. Evans, J. C., Jackson, S. K., Rowlands, C. C. & Barratt, M. D. (1984) Biochim. Biophys. Acta **792**, 239–242
33. Evans, J. C., Jackson, S. K., Rowlands, C. C. & Barratt, M. D. (1985) Biochim. Biophys. Acta **835**, 421–425
34. Evans, J. C., Rao, K. R. N., Jackson, S. K., Rowlands, C. C. & Barratt, M. D. (1985) J. High Resolut. Chromatogr. Chromatogr. Commun. **8**, 829–830
35. Li, A. S. W. & Chignell, C. F. (1987) Photochem. Photobiol. **45**, 191–197
36. Arroyo, C. M., Kramer, J. H., Leiboff, R. H., Mergner, G. W., Dickens, B. F. & Weglicki, W. B. (1987) Free Radical Biol. Med. **3**, 313–316
37. Feix, J. B. & Kalyanaraman, B. (1989) Biochim. Biophys. Acta **992**, 230–235

Received 9 October 1990/7 December 1990; accepted 2 January 1991