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Interrelation between nucleotide degradation and aldehyde formation in red blood cells

Influence of xanthine oxidase on metabolism: an application of nucleotide and aldehyde analyses by high-performance liquid chromatography

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ABSTRACT

The mechanism by which hypoxia leads to irreversible cellular damage is poorly understood. A decrease in purine nucleotides is common to all ischaemic tissues, yielding hypoxanthine as the substrate of the xanthine oxidase reaction. Excessive production of radicals via xanthine oxidase induces peroxidation of unsaturated fatty acids, accompanied with the formation of aldehydes. The nucleotides and aldehydes were determined by high-performance liquid chromatography (HPLC) of red blood cell extracts. Nucleotides and their derivatives were determined by HPLC on an ODS column and elution with 10 mM phosphate buffer containing 2 mM *tert.*-butylammonium phosphate. The aldehyde production in glucose deprived red blood cells was stimulated by addition of xanthine oxidase and by inhibition of different haemotype enzymes with sodium azide. Aldehydes were analysed by derivatization to dinitrophenylhydrazones, followed by thin-layer chromatographic and HPLC separation with aqueous methanol on an ODS column. The HPLC methods presented are appropriate for the determination of nucleotides, nucleosides and nucleobases, in addition to alkenals and hydroxyalkenals in extracts of oxidatively stressed red blood cells.

INTRODUCTION

There are a wide range of factors relating the metabolic state of erythrocytes to their susceptibility to oxidative stress. It is known that depletion of erythrocyte adenosine triphosphate before incubation under radical-generating conditions favours accumulation of malondialdehyde (MDA)[1]. The peroxidative susceptibility seems to be determined by the metabolic state of the cell and the site of the peroxidized lipids within intracellular pools. A decrease in purine nucleotides is common to all ischaemic tissues[2–4].

It has been proposed that oxygen radicals are generated via xanthine oxidase reactions in reoxygenated tissues[5,6]. Hypoxanthine, the substrate of the xanthine oxidase reaction, accumulates during oxygen deficiency owing to the breakdown of purine nucleotides[7]. The conversion to uric acid via xanthine oxidase induces lipid peroxidation of polyunsaturated fatty acids[8]. Lipid peroxidation results in a wide range of carbonyl products, some of which are extremely reactive[9,10]. Aldehydes are formed as secondary oxidation products from the primary products of hydroperoxides. It has been known for many years that some of these aldehyde products inhibit energy-requiring processes of the cell[9,10]. An important representative of the aldehydes generated is 4-hydroxy-2-nonenal (4-HNE), occurring in well known biological model systems for oxidative burst (e.g., NADPH oxidation by liver microsomes, carbon tetrachloride intoxication). The aldehyde production could be used for the assessment of the oxidative loading of cells. Under ischaemic and post-ischaemic conditions, radicals are generated via xanthine oxidase by conversion of purine breakdown products. Therefore, under these conditions it is useful to determine both parameters (nucleotides and aldehydes) in order to evaluate the radical-induced changes. Both phenomena, nucleotide degradation and lipid peroxidation, could be analysed by high-performance liquid chromatography (HPLC).

The nucleotides, especially ATP, are of importance for proteolytic pathways occurring in red blood cells (ATP increases the degradation of untreated proteins 4–6-fold in reticulocyte extracts). The share of ATP-independent proteolytic pathways which may give protection against the accumulation of proteins damaged by oxygen radicals or other active oxygen species is to be determined[11]. As a consequence of the production of free radicals ($O_2^{-\cdot}$), haemoglobin in red blood cells is first oxidized to methaemoglobin and then to hemicrome[12]. At the same time, modifications of the membrane proteins, lipids, cell permeabilities and autologous IgG binding have been reported. The final result of these events is cell lysis and/or phagocytosis. Both processes occur in vivo. Owing to the heterogeneity of the physico-chemical properties of polar or apolar compounds (nucleotides, nucleosides) and the very different concentrations (low concentration of nucleobases), a reversed-phase (RP) ion-pair HPLC separation was applied to measure the breakdown of nucleotides and the increase in nucleosides and nucleobases in a single run. Red blood cells of rabbits were used in the HPLC methods for the determination of nucleotides and aldehydes. The aldehyde production in red blood cells (RBC) was initiated by addition of xanthine oxidase (XOD) and further stimulated by addition of sodium azide, which inhibits different haemotype enzymes (e.g., catalase), leading to the acceleration of radical generation.

EXPERIMENTAL

Determination of nucleotides
    Chemicals. All reference standard of the highest analytical grade available were purchased from Boehringer (Mannheim, F.R.G.) and Sigma (St. Louis, MO, U.S.A.). $NH_4H_2PO_4$ was purchased from Fisher scientific (Fairlawn, NJ, U.S.A.), acetonitrile from Merck (Darmstadt, F.R.G.), TBA (tetratbutylammonium phosphate, PIC reagent A) from Waters Assoc. (Milford, MA, U.S.A.) and XOD from Boehringer.

Cell preparation. Red blood cells were drawn from the ear vein of rabbits, washed twice in an isotonic solution of triethanolamine buffer (pH 7.43) and incubated at 37°C with XOD (0.2 units per 10-ml reaction volume) and sodium azide (2 mmol/l).

Extraction procedure and sample preparation. The samples were deproteinized (6% perchloric acid), centrifuged (8 min 1200 *g*) and neutralized with triethanolamine (1 mol/l)–potassium carbonate (1.3 mol/l) (pH 9). After filtration, 50 μl of the supernatant were analysed by HPLC.

HPLC instrument and chromatographic conditions. A Perkin-Elmer (Norwalk, CT, U.S.A.) liquid chromatograph was used, consisting of a M410 pump system, LC-95 variable-wavelength detector (operated at 254 nm), LCI-100 integrator and a Rheodyne injector. The column was Supelcosil LC-18-S (5 μm) (150 × 4.6 mm I.D.) with 9800 theoretical plates, with a 25 mm × 4.6 mm I.D. precolumn.

Buffer A was 0.01 *M* $NH_4H_2PO_4$ containing 2 m*M* PIC A and buffer B was 80% buffer A containing 20% (v/v) acetonitrile, with the following gradient: 5 min linear gradient from 100% A to 70% B, 8 min isocratic 70% B–30% A, 20 min convex gradient to 100% B (curve 3; this curve leads to 37% B after 10 min and to 65% B after 15 min of this gradient elution), 7 min isocratic 100% B, 2 min to 100% A; at the end the system was switched back by pumping buffer A for 10 min. The flow-rate was 1 ml/min. Peak identification and quantification were performed as described elsewhere[13].

Determination of aldehydes

Chemicals. Aldehyde standards (dinitrophenylhydrazones) were obtained from Professor H. Esterbauer (University of Graz, Austria). The solvents *n*-hexane, dichlormethane and methanol and thin-layer chromatographic (TLC) plates (silica gel 60 F_{254}, 0.2 mm thickness) from Merck (Darmstadt, F.R.G.) were used. 2,4-Dinitrophenylhydrazine (DNPH) was obtained from Union Chimique (Brussels, Belgium), dissolved in a 1 *M* hydrochloric acid, extracted with 15 ml of *n*-hexane, then adjusted to 1.8 m*M* solution by absorbance measurement.

Preparation of 2,4-dinitrophenylhydrazones from biological samples[14,15]. After incubation of 4.5 ml of a suspension of RBC (+ 0.5 ml of EDTA) with 5 ml of DNPH solution for 2 h in the dark with mixing, samples were kept for 1 h in an ice-bath in the dark, then extracted with 7 ml of dichloromethane, centrifuged at 900 *g* (three times), evaporated to dryness and the residue was dissolved in 1 ml of dichloromethane.

Thin-layer chromatography. TLC plates were developed with dichloromethane in comparison with known standards[14]. Two individual fractions were scraped off [zone I, zone III according to ref. 14, containing 4-hydroxyalkenals (I) and alkenals, 2-alkenals and ketones (III)], extracted with 10 ml of methanol (three times), evaporated to dryness and the residue was dissolved in 1 ml of methanol.

HPLC instrument and chromatographic conditions. A Shimadzu LC-6A HPLC pump, a Rheodyne injector and a Hewlett-Packard 104A photodiode-array detector with an HP 9000 Series 300 Workstation (9153A Winchester drive, Think-Jet printer, Color Pro plotter) were used. The eluent was methanol–water (4:1, v/v) at a flow-rate of 0.9 ml/min. The column was Nucleosil $5C_{18}$ (Macherey, Nagel &Co., Düren, F.R.G.) (250 × 4.0 mm I.D.) with a 50 × 4.0 mm I.D. precolumn.

Peak identification was performed using internal standards and comparison

with the spectra of reference compounds. Quantification was achieved by separating authentic standards under identical conditions. Recoveries were determined for all reference compounds following derivatization and TLC.

RESULTS AND DISCUSSION

Nucleotide determination

The gradient RP-HPLC method with ion pairing applied to RBC extracts allowed the separation of hypoxanthine, xanthine, uric acid, inosine, guanosine, adenine, adenosine, NAD, IMP, AMP, GDP, ADP, GTP and ATP (Fig. 1). The separation of nucleotides, nucleosides and nucleobases obtained was comparable to those reported by other workers[16,17]. The low-concentrated hypoxanthine, xanthine and uric acid were eluted in the first few minutes of separation without peak broadening. The method allowed changes in nucleotide metabolism to be monitored during incubation experiments with increased nucleotide catabolism induced by glycose starvation, and also the influence of radical formation on nucleotide metabolism. The radical formation in ischaemic tissues via purine breakdown could be assessed, as radical formation via XOD is the most important source during and following oxygen deficiency[6].
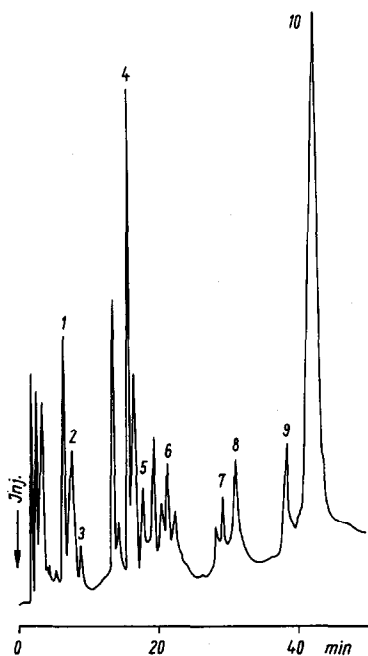

Fig. 1. Ion-pair RP-HPLC separation of nucleobases and nucleotides extracted from RBC using a gradient system (see Experimental). Eluents: (A) 10 mmol/l $NH_4H_2PO_4$ with 2 mmol/l tetrabutylammonium phosphate and (B) eluent A containing 20% (v/v) acetonitrile. Flow-rate, 1 ml/min. A Supelcosil LC-18-S (5 μm) column (150 × 4.6 mm I.D.) was used. Detection wavelength, 254 nm (0.02 a.u.f.s.). Peaks: 1 = hypoxanthine; 2 = xanthine; 3 = uric acid; 4 = NAD^+; 5 = IMP; 6 = AMP; 7 = GDP; 8 = ADP; 9 = GTP; 10 = ATP. Peak identities of the biological sample were confirmed by coelution with reference compounds.

Glucose starvation yielded an increased catabolism of purine nucleotides in red cells[18]. Hypoxanthine accumulated under these conditions. The addition of XOD yielded a radical-generating system and furthermore accelerated the degradation of ATP, ADP and AMP in comparison with controls without addition of XOD to the suspension. RBC in a control experiment with glucose-containing buffer showed no degradation of adenine nucleotides[19]. The addition of sodium azide had no significant effect on nucleotide breakdown but modified the response of the lipid peroxidation in the RBC. A comparison of nucleotide degradation under glucose deprivation and glucose deprivation with added XOD yielded a faster decrease in adenine nucleotides in the latter instance.

The ion-pair RP-HPLC separation of nucleobases, nucleosides and nucleotides is influenced by the interaction of the cationic ion-pair reagent with the ionic nucleotides. The formation and retention of ion pairs causes the sequence of elution of nucleoside monophosphates before nucleoside diphosphates and before nucleoside triphosphates. The buffer salt concentration of 10 mM $NH_4H_2PO_4$ allowed no competition with the nucleotides for the ion-pair reagent, and maintained a sufficient amount of free ion pair cations[20].

Aldehyde determination

After derivatization of free aldehydes with 2,4-DNPH, the extraction yielded a separation of polar and non-polar fractions[14]. The non-polar fraction contained *n*-alkenals, 2-alkenals, dicarbonyl compounds and 4-hydroxyalkenals. Zone I contained 4-hydroxynonenal, 4-hydroxyoctenal and 4-hydroxyhexenal. Fig. 2 (bottom) shows the chromatogram of OH-alkenal standards obtained after TLC. The upper panel shows the spectrum of the dinitrophenylhydrazone of 4-hydroxyhexenal and the corresponding standard compound.

Zone III consists of pentanal, 2,4-hexadienal, hexanal, 2,4-heptadienal, octenal, 2,5-nonadienal and 2,4 decadienal. The chromatogram is shown in Fig. 3 (bottom).
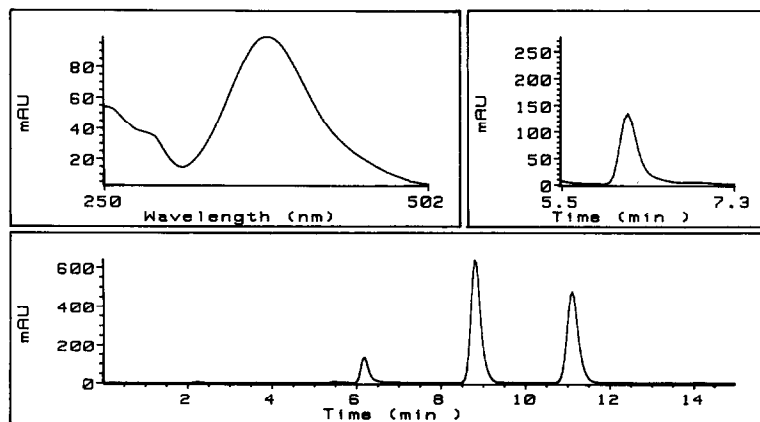

Fig. 2. (Bottom) chromatogram of a standard mixture of 2,4-dinitrophenylhydrazones of hydroxyalkenals (zone I after TLC). Retention times: 6.3 min = 4-hydroxyhexenal (also in the inset in the upper panel, spectrum of 4-hydroxyhexenal), 8.8 min = 4-hydroxyoctenal, 11.3 min = 4-hydroxynonenal. Column, Nucleosil 5C$_{18}$ (250 × 4.0 mm I.D.); flow-rate, 0.9 ml/min.
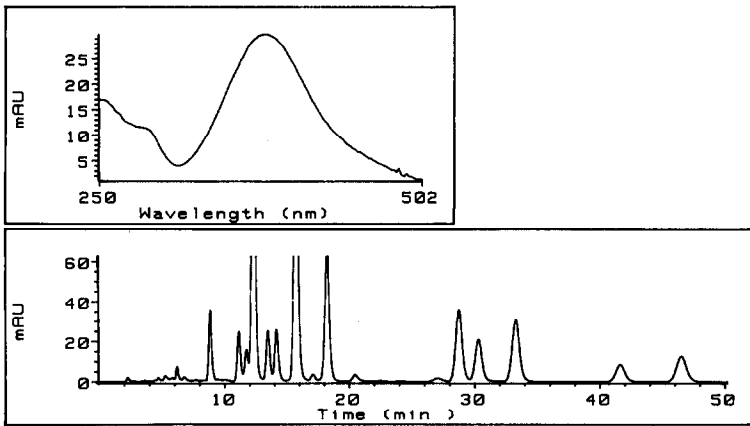
Fig. 3. (Bottom) chromatogram of a standard mixture of 2,4-dinitrophenylhydrazones of aldehydes (zone III after TLC) and (top) spectrum of 2,4-heptadienal. Retention times: 12.5 min = pentanal; 16 min = 2,4-hexadienal; 18.4 min = hexanal; 28.5 min = 2,4-heptadienal; 30.5 min = octenal; 33.5 min = 2,5 nonadienal; 46.5 min = 2,4-decadienal).

In the upper panel the spectrum of the dinitrophenylhydrazone of 2,4-heptadienal is shown. After addition of XOD and hypoxanthine to the red cell suspension containing sodium azide as inhibitor of haemotype enzymes the first sample was taken and prepared for aldehyde analysis (extraction and TLC). Fig. 4 shows the chromatogram of zone I after TLC of the lipid peroxidation products. The chromatogram in Fig. 5 (bottom), was obtained after 60 min of incubation with XOD and hypoxanthine (sodium azide). The upper panel shows the spectra of the dinitrophenylhydrazones of 4-hydroxynonenal from the standard chromatogram and the chromatogram of the biological extract comparison which were used for peak identification in the extracts of the cell suspensions. 4-HNE production was much more pronounced after addition
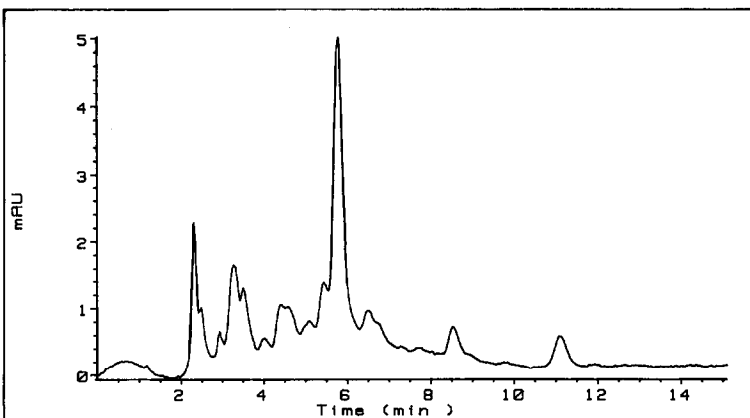

Fig. 4. Chromatogram of zone I after TLC of lipid peroxidation products originating from RBC immediately after addition of XOD to the suspension containing hypoxanthine and sodium azide (initial value of 4-hydroxynonenal at retention time 11 min).
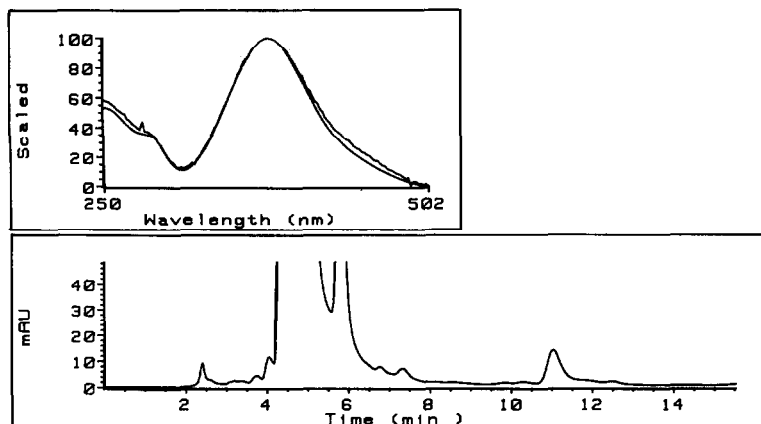
Fig. 5. (Bottom) chromatogram of zone I after TLC of lipid peroxidation products of RBC incubated with XOD, hypoxanthine and sodium azide (after 60 min at 37°C). (Top) spectra for comparison of 4-hydroxy-nonenal from the biological extract and a standard compound.

of XOD and hypoxanthine (250 μmol/l) compared with the incubation with XOD only, using the hypoxanthine from the ATP degradation under glucose deprivation conditions. Nucleotide analysis indicated an increase in hypoxanthine from 10 μmol/l at the beginning of the incubation experiment (after glucose-free washing of RBC suspension) to 300 μmol/l after incubation for 4 h without glucose. The incubation of the RBC suspension with XOD alone yielded a 25-fold increase in 4-HNE (17.94 ± 2.9 μmol/l) and a 150-fold increase for the glucose-free incubation of RBC with XOD and hypoxanthine (107.6 ± 17.4 μmol/l).

The ATP degradation in RBC was accelerated in comparison with control incubations without detectable 4-HNE formation (glucose-free incubation from 1.3 ± 0.08 to 1.05 ± 0.05 mmol/l, glucose-free + XOD to 0.84 ± 0.06 mmol/l, glucose-free + XOD + hypoxanthine to 0.9 ± 0.08 mmol/l; $n=4$). Fig. 6 shows the chromatogram of an extract of RBC suspension after 60 min of lipid peroxidation induced by XOD and hypoxanthine. Pentanal, hexanal and 2,4-decadienal were increased (2,4-decadienal is shown in the inset in Fig. 6) in comparison with the initial values 20-, 26- and 15-fold (1.78 ± 0.27, 2.43 ± 0.25, 1.93 ± 0.25 μmol/l), respectively, after incubation of RBC for 4 h with XOD + Hyp. Sodium azide was applied in all experiments to stimulate the lipid peroxidation in RBC containing very active antioxidant enzymes, such as superoxide dismutase, catalase or glutathione peroxidase. Reactive oxygen species could diffuse across the RBC membrane.

In RBC exposed to hypoxanthine and XOD, which generate both superoxide and hydrogen peroxide, the lipid peroxidation and degradation of proteins to free amino acids increase dramatically owing to deterioration of membrane organization and an increase in proteolytic processes induced by aldehyde-mediated reactions[11]. This effect coincides with the appearance of methaemoglobin and other oxidized forms of haemoglobin in the cells. The simplest explanation of these observations is that the major cell protein haemoglobin is damaged either by the oxygen radicals ($HO_2\cdot$, $O_2^{-}\cdot$, $\cdot OH$), or by other activated oxygen species (H_2O_2 or possibly $^1O_2$) generated by hypoxanthine + XOD. For the determination of the radical-induced
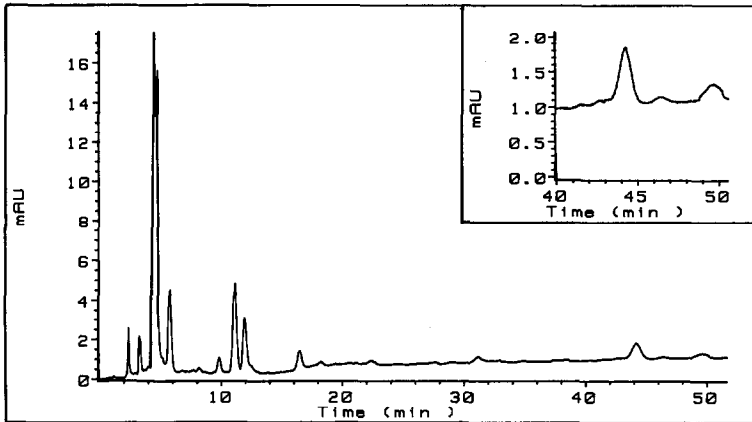
Fig. 6. Chromatogram of zone III after TLC of lipid peroxidation products of RBC incubated with xanthine oxidase, hypoxanthine and sodium azide (after 60 min at 37°C). Retention times: 12.5 min = pentanal; 16.5 min = hexanal; 44.5 min = 2,4-decadienal (detailed in the inset). Peaks were identified by comparison of spectra with standard compounds.

lipid peroxidation the TLC–HPLC determination of aldehydes was applied. The detectable amount of lipid peroxidation measured by generation of 4-HNE increased during the 4 h of the incubation of rabbit erythrocytes at 37°C. The appearance of lipid peroxidation was followed by haemolysis. Glucose could protect against lipid peroxidation by maintaining normal levels of reduced glutathione. The incubation with hypoxanthine and XOD generates larger amounts of urate, which has been shown to protect membrane lipids against peroxidation[21].

The aldehyde detected in the experiments could be generated by cleavage reactions from primary hydroperoxides or from secondary or tertiary oxygenated products. Hexanal can be formed by dismutation of various monohydroperoxides (e.g., 15-hydroperoxyarachidonic acid) and 4-hydroxynonenal in peroxidizing liver microsomes arises exclusively from arachidonic acids of polar lipids[8]. This could also be due to RBC peroxidation. The measured increase in 4-HNE is of interest because of the inhibiting effects on a number of cellular functions (cellular glycolysis, 5'-nucleotidase, adenylate cyclase)[22].

The chromatographic methods presented allow the assessment of radical-induced changes in cells by the determination of changes in nucleotide metabolism and of the generation of lipid peroxidation products, and could therefore be applied to various cells and tissues showing changes after the action of reactive oxygen species.

REFERENCES

1 D. W. Allen and J. J. Cierzan, J. Lab. Clin. Med., 112 (1988) 87.
2 D.A. Hems and J. T. Brosnan, Biochem. J., 120 (1970) 105.
3 M.-F. Vincent, G. van den Berghe and H. G. Hers, Biochem. J., 202 (1982) 117.
4 S. Marubayashi, M. Takenaka, K. Dohi, H. Ezaki and T. Kawasaki, Transplantation, 30 (1980) 294.
5 D. N. Granger, G. Rutili and J. M. McCord, Gastroenterology, 81 (1981) 22.
6 J. M. McCord and R. S. Roy, Can. J. Physiol. Pharmacol., 60 (1982) 1346.

7 H. Osswald, *Pflügers Arch.*, 371 (1977) 45.
8 A. Benedetti and M. Comporti, *Bioelectrochem. Bioenerg.*, 18 (1987) 187.
9 E. Schauenstein, G. Schatz and G. Benedikt, *Monatsh. Chem.*, 92 (1961) 442.
10 E. Schauenstein and H. Esterbauer, *Monatsh. Chem.*, 94 (1963) 164.
11 K. J. A. Davies and A. L. Goldberg, *J. Biol. Chem.*, 262 (1987) 8227.
12 J. H. Jandl, L. K. Engle and D. W. Allen, *J. Clin. Invest.*, 39 (1960) 1818.
13 A. Werner, W. Siems, G. Gerber, H. Schmidt, S. Gruner and H. Becker, *Chromatographia*, 25 (1988) 237.
14 H. Esterbauer, K. H. Cheeseman, M. U. Dianzani, G. Poli and T. F. Slater, *Biochem. J.*, 208 (1982) 129.
15 G. Poli, M. U. Dianzani, K. H. Cheeseman, T. F. Slater, J. Lang and H. Esterbauer, *Biochem. J.*, 227 (1985) 629.
16 D. R. Webster, G. D. Boston and D. M. Paton, *J. Liq. Chromatogr.*, 8 (1985).
17 V. Stocchi, L. Lucchiarini, F. Canestrari, M. P. Piscentini and G. Fornaini, *Anal. Biochem.*, 167 (1987) 181.
18 F. Bontemps, G. van den Berghe and H. G. Hers, *J. Clin. Invest.*, 77 (1986) 824.
19 A. Werner, I. Rapoport and G. Gerber, *Biomed. Biochim. Acta*, 46 (1987) 324.
20 D. Perrett, in C. K. Lim (Editor), *HPLC of Small Molecules—A Practical Approach*, IRL Press, Oxford, 1986, p. 221.
21 A. Sevanian, S. F. Muakkassah-Kelly and S. Montestruque, *Arch. Biochem. Biophys.*, 223 (1983) 441.
22 H. Esterbauer, in D. C. H. McBrien and T. F. Slater (Editors), *Free Radicals, Lipid Peroxidation and Cancer*, Academic press, London, 1982, p. 101.