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DETECTION BY FLUORESCENCE OF PEROXIDES AND CARBONYLS IN SAMPLES OF ARACHIDONIC ACID

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1 Products of lipid peroxidation were compared in two different grades of commercially-obtained arachidonic acid.

2 The less pure 90% sample was yellow in colour, had low reactivity with 2-thiobarbituric acid and diene conjugation but high u.v. fluorescence, whereas the 99% pure sample, which was clear in colour, showed a reverse pattern of oxidation products.

3 The significance of these findings is discussed.

Introduction

Oxidation of polyunsaturated fatty-acids (PUFA) can take place by enzyme catalysed reactions as well as non-enzymatic autoxidation. Lipid autoxidation is an autocatalytic process initiated and sustained by free radicals. During the process of lipid autoxidation numerous primary peroxidic and secondary carbonyl compounds are formed (Noble & Nawar, 1971: Gutteridge, Lamport & Dormandy, 1976). These molecules, derived in this way, are known to possess potent biological properties demonstrated by their antimicrobial, anti-cancer, platelet aggregating, blood coagulating and chemotactic effects (Schauenstein, 1967; Mickel & Horbar, 1974; Barrowcliffe, Gutteridge & Dormandy, 1975; Turner, Campbell & Lynn, 1975; Gutteridge *et al.*, 1976).

Recently, enzyme catalysed pathways of PUFA oxidation, such as those leading to prostaglandin and leukotriene formation, have been studied in detail. As a result, it is now recognized that enzymic and non-enzymic pathways of PUFA oxidation share common intermediates such as the cyclic endoperoxides and possibly free radicals as well (Pryor, Stanley & Blair, 1976; Kuel, Humes, Egan, Ham, Beveridge & Van Arman, 1977). In both types of oxidation, reactivity with 2-thiobarbituric acid (TBA) has been usefully applied as a measure of change in the fatty-acid molecule.

Arachidonic acid is frequently added to experimental systems, such as platelets, for the study of cyclo-oxygenase and lipoxygenase activities. When undertaking such experiments it is desirable to know the extent to which lipid autoxidation has taken place, and hence the possible degree of biological activity intrinsic to that sample.

This paper illustrates the variability of lipid autoxidation products seen in commercial samples of arachidonic acid and suggests simple procedures by which these patterns can be determined. Part of this work was presented as a poster to the British Pharmacological Society (Gutteridge & Kerry, 1981).

Methods

Measurement of thiobarbituric acid reactivity

Samples of arachidonic acid were diluted 1 in 800 with 0.15 M NaCl/0.024 M phosphate buffer pH 7.4; 1.0 ml of this dilution was added to an equal volume of potassium hydrogen phthalate-HCl buffer (pH 3.5) followed by 1.0 ml 1.0% w/v thiobarbituric acid (TBA) in 0.05 M NaOH (Gutteridge, 1982). The tubes were heated for 15 min at 100°C, cooled and the absorbance read at 532 nm.

Measurement of fluorescence

Arachidonic acid (10 μl) was dissolved in 1.0 ml methanol and the fluorescence measured with a Perkin-Elmer model MPF 4 spectrofluorimeter using a block standard containing 10^{-7} M tetraphenylbutadiene as the reference standard. This standard was set to 100 units with the following instrument settings: excitation (ex.) 350 nm, emission (em.) 440 nm, filter 430 nm, slits 10 nm and sensitivity x 1. Relative fluorescence intensity (RFI) of the arachidonic acid samples was measured at ex. 340 nm, em. 390 nm, filter 350 nm, slits 10 nm and sensitivity x 3, for u.v. fluorescence and for visible fluorescence, ex. 360 nm, em. 430 nm, filter 390 nm, slits 10 nm and sensitivity x 3. The RFI value of methanol under these conditions was 4 and 2 units

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respectively. These were subtracted from the test values shown in Table 1.

Measurement of u.v.-absorption (diene conjugation)

Arachidonic acid 0.1 ml was diluted 1 in 2,000 with methanol and u.v. absorption scan difference spectra taken using a methanol blank. Absorbance at 235 nm was recorded as a measure of diene conjugation of lipid peroxides.

Decomposition of lipid peroxides by heating

Arachidonic acid (10 μl) was heated at 100°C for 15 min in tightly capped glass tubes. After cooling, 1.0 ml methanol was added and the fluorescent properties determined as described above.

Materials

Arachidonic acid grade IV and grade I were obtained from the Sigma Chemical Company, Poole, Dorset. All other chemicals were of Analar grade, where available, and were obtained from BDH Ltd., Poole, Dorset. Analyses were performed on the arachidonic acid samples immediately after opening the ampoule, following storage at − 20°C.

Results

Analysis of two samples of graded arachidonic acid showed them to contain entirely different patterns of lipid peroxidation products (Table 1). The grade I sample, claimed to be 99% pure, was clear in appearance but contained substantially higher levels of conjugated dienes (lipid peroxides) on the basis of u.v. absorbance than the yellow grade IV sample claimed to be 90% pure. The presence of lipid peroxides was confirmed by the greater TBA-reactivity detected in the 99% pure sample. The less pure (90%) arachidonic acid had lower levels of lipid peroxides but substantially higher autofluorescence (Table 1). Accelerated degradation of the peroxides, present in the 99% pure sample, either in the presence or absence of dioxygen greatly enhanced the formation of fluorescent material (Table 1).

Discussion

Ultraviolet and visible fluorescence observed in autoxidized PUFA has been tentatively ascribed to the presence of endoperoxides and the formation of carbonyl polymers (Gutteridge, Lunec & Heys, 1978; Gutteridge & Kerry, 1981; Gutteridge *et al.*, 1982).

When two different grades of commercially-obtained arachidonic acid were examined for intrinsic autoxidation products, the less pure (90%) sample was yellow in colour, had low TBA-reactivity and diene conjugation but high u.v. fluorescence. The 99% pure sample was colourless and showed a reverse pattern of oxidation products. TBA-reactivity is interpreted here as a measure of lipid peroxides present in the arachidonic acid sample, which decompose during the acid-heating stage of the TBA-test to liberate malondialdehyde (MDA).

The lower levels of TBA-reactivity and diene con-

Table 1 Lipid autoxidation products in two commercial grades of arachidonic acid

Grade of arachidonic acid	*Appearance*	*TBA-reactivity* (A 532 nm)	*Diene conj.* (A 235 nm)	*Relative fluorescence units × 3* (Ex. 340 nm, Em. 390 nm)	(Ex. 360 nm, Em. 430 nm)
Grade IV (90% pure)	Yellow	0.10	0.12	105	40
Grade I (99% pure)	Clear	0.65	0.35	10	8
Changes in fluorescence after decomposing the lipid peroxides by heating in O_2 and N_2					
Grade I (99% pure) Heated in N_2				144	216
Grade I (99% pure) Heated in O_2				123	294

The results presented are a mean of two separate measurements, which were found to be reproducible.

jugation but higher levels of fluorescent material found in the 90% pure sample could be explained by the following mechanism: arachidonic acid peroxides formed during the isolation and purification of the fatty-acid are sealed in the ampoule under nitrogen during packaging. The slow decomposition of these peroxidic intermediates, which is not dioxygen dependent, takes place during subsequent storage. Thus TBA-reactivity and diene conjugation can decrease as secondary carbonyls and fluorescence increase.

Accelerated decomposition of lipid peroxides, by heating, has previously been shown to increase fluorescence in autoxidized PUFA; fluorescence being attributed to the polymerization of carbonyl compounds, including MDA (Gutteridge, Kerry & Armstrong, 1982). Here, it was also observed that a sample with a high lipid peroxide content could be decomposed to carbonyl products either in the presence or absence of dioxygen. During the process of polymerization it has previously been observed that MDA loses substantial amounts of its TBA-reactivity (Gutteridge, 1975).

Autoxidized arachidonic acid has a potent prostaglandin-like activity (Gutteridge, 1974) and this can be attributed to the formation of cyclic endoperoxides similar to the prostaglandin endoperoxides of the cyclo-oxygenase pathway (Pryor *et al.*, 1976). Before adding arachidonic acid to experimental systems it would seem desirable, if not essential, to determine the pattern of autoxidation products present in the sample. The methods outlined in this paper have proved useful in our hands for such a purpose.

References

BARROWCLIFFE, T.W., GUTTERIDGE, J.M.C. & DORMANDY, T.L. (1975). The effect of fatty acid oxidation products on blood coagulation. *Thromb. Diath. Haemorrh.*, **33,** 271–277.

GUTTERIDGE, J.M.C. (1974). *Consequences of Lipid Autoxidation in Biological Material.* Ph.D. Thesis, University of London.

GUTTERIDGE, J.M.C. (1975). The use of standards for malondialdehyde. *Analyt. Biochem.*, **69,** 518–526.

GUTTERIDGE, J.M.C. (1982). Free radical damage to lipids, amino acids, carbohydrates and nucleic acids determined by thiobarbituric acid-reactivity. *Int. J. Biochem.* (in press).

GUTTERIDGE, J.M.C. & KERRY, P.J. (1981). Autofluorescence associated with fatty-acid peroxides. *Br. J. Pharmac.*, **74,** 918–919P.

GUTTERIDGE, J.M.C., KERRY, P.J. & ARMSTRONG, D. (1982). Autoxidised and lipoxidase treated polyunsaturated fatty-acids. Autofluorescence associated with the decomposition of lipid peroxides. *Biochim. biophys. Acta* (in press).

GUTTERIDGE, J.M.C., LAMPORT, P. & DORMANDY, T.L. (1976). The antibacterial effect of water-soluble compounds from autoxidising linolenic acid. *J. med. Microbiol.*, **9,** 105–110.

GUTTERIDGE, J.M.C., LUNEC, J. & HEYS, A.D. (1978). The detection of native fluorescence in peroxidised fatty-acids. *Analyt. Lett.*, **7,** 537–544.

KUEL, F.A., HUMES, J.L., EGAN, R.W., HAM, E.A., BEVERIDGE, G.C. & VAN ARMAN, C.G. (1977). Role of prostaglandin endoperoxide PGG_2 in inflammatory processes. *Nature, Lond.*, **265,** 170–172.

MICKEL, H.S. & HORBAR, J. (1974). The effect of peroxidised arachidonic acid upon human platelet aggregation. *Lipids*, **9,** 68–71.

NOBLE, A.C. & NAWAR, W.W. (1971). The autoxidation of highly unsaturated fatty-acids: methyl-4,7,10,13,16,19-docosahexaenoate. *J. Am. Oil Chem.Soc.*, **48,** 800–803.

PRYOR, W.A., STANLEY, J.P. & BLAIR, E. (1976). Autoxidation of polyunsaturated fatty-acids. II: A suggested mechanism for the formation of TBA-reactive materials from prostaglandin-like endoperoxides. *Lipids*, **11,** 370–379.

SCHAUENSTEIN, E. (1967). Autoxidation of polyunsaturated esters in water: chemical structure and biological activity of products. *J. Lipid Res.*, **8,** 417–428.

TURNER, S.R., CAMPBELL, J.A. & LYNN, W.S. (1975). Polymorphonuclear leucocyte chemotaxis towards oxidized lipid components of cell membranes. *J. exp. Med.*, **141,** 1437–1441.

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