

Free Radical-Mediated Lipid Peroxidation in Cells: Oxidizability Is a Function of Cell Lipid *bis*-Allylic Hydrogen Content[†]

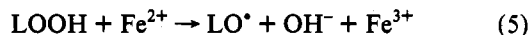
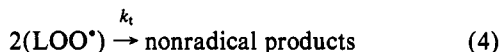
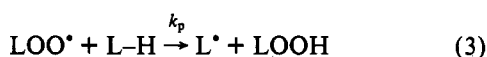
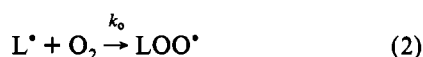
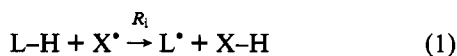
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Received November 9, 1993; Revised Manuscript Received February 22, 1994*

ABSTRACT: Oxidizability of lipids in homogeneous solution varies linearly with the extent of their unsaturation. *In vitro* cellular, as well as *in vivo*, studies of oxidizability have generally relied upon chemical indicators of peroxidation such as thiobarbituric acid-reactive substances. To examine the oxidizability of lipids in cells, we have measured oxygen uptake and, using electron paramagnetic resonance spin trapping with α -(1-oxo-4-pyridyl)-*N*-*tert*-butylnitron (POBN), the real time generation of lipid-derived free radicals. We have used our experimental *in vitro* cellular lipid modification model to examine the rate and extent of lipid peroxidation versus the degree of lipid unsaturation in L1210 murine leukemia cells. Lipid peroxidation was stimulated using the prooxidants iron, ascorbate, and the ether lipid compound 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine. We did a total cellular lipid analysis to determine the number of lipid carbon–carbon double bonds contained in L1210 cells enriched with eight fatty acids of different degrees of unsaturation. We found in cellular lipids that (i) lipid chain length had no apparent effect on the rate or extent of radical formation; (ii) the maximum amount of lipid radical generated increases with the total number of *bis*-allylic positions in the cellular lipids; and, most importantly, (iii) the rate of cellular lipid peroxidation increases exponentially with the number of *bis*-allylic positions. Our quantitative results clearly demonstrate, for the first time, that the number of *bis*-allylic positions contained in the cellular lipids of intact cells determines their susceptibility, *i.e.*, oxidizability, to free radical-mediated peroxidative events.

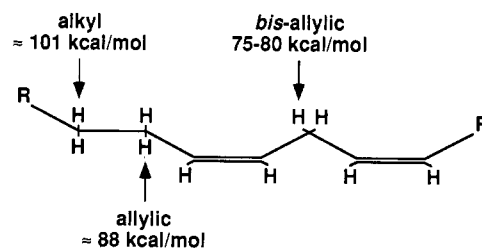
Lipid peroxidation is a free radical process comprised of three principal events (Porter, 1984; Gardner, 1989; Barclay, 1993): initiation (reaction 1), a propagation cycle (reactions 2 and 3), and termination (reaction 4)



where L–H represents a lipid, generally a polyunsaturated fatty acid moiety (PUFA),¹ and R_i is the rate of initiation. Initiation (reaction 1) occurs when a highly energetic one-electron oxidant ($\text{X}^{\bullet}$), such as a hydroxyl radical, abstracts a hydrogen atom from a lipid fatty acid chain, producing a

carbon-centered radical, $\text{L}^{\bullet}$. This lipid radical rapidly reacts with dioxygen ($k_o = 3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) (Hasegawa & Patterson, 1978; Buettner, 1993), producing a lipid peroxy radical, $\text{LOO}^{\bullet}$ (reaction 2). $\text{LOO}^{\bullet}$ is the chain-carrying radical of the propagation cycle (reaction 3), $k_p \approx 10 \text{ M}^{-1} \text{ s}^{-1}$ (Buettner, 1993). The lipid hydroperoxides formed can serve as precursors to additional initiation or chain-branching events, *e.g.*, reaction 5. Thus, lipid peroxidation is a complex free radical, branching-chain reaction.

The rate of the propagation event is governed by the various carbon–hydrogen bond dissociation energies along the lipid chain. The weakest carbon–hydrogen bonds are those at the *bis*-allylic methylene positions, 75–80 kcal/mol bond dissociation energy versus $\approx 88 \text{ kcal/mol}$ for a monoallylic hydrogen, and $\approx 101 \text{ kcal/mol}$ for an alkyl C–H bond (Gardner, 1989; Koppenol, 1990):



The energetics of reaction 3 favor attack by $\text{LOO}^{\bullet}$ at the *bis*-allylic methylene hydrogens of the unsaturated lipids (Gardner, 1989; Buettner, 1993; Koppenol, 1990); thus,

[†] This investigation was supported by Grant CA31526 awarded by the National Cancer Institute, by Grant HL49264 awarded by the National Heart, Lung, and Blood Institute, Department of Health and Human Services, and by The Art Ehrmann Cancer Fund, Fraternal Order of Eagles. Data analysis utilized the Clinfo system, Grant RR59 from the General Clinical Research Centers Program, Division of Research Resources, NIH.

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• Abstract published in *Advance ACS Abstracts*, April 1, 1994.

¹ Abbreviations: DBI, double bond index (the mean number of double bonds per fatty acid); EPR, electron paramagnetic resonance; FBS, fetal bovine serum; LOOH, lipid hydroperoxide; MBI, methylene bridge index (the mean number of *bis*-allylic methylene positions per fatty acid); POBN, α -(1-oxo-4-pyridyl)-*N*-*tert*-butylnitron; PUFA, polyunsaturated fatty acid.

theoretically the more *bis*-allylic methylene positions there are in a lipid, the greater will be its oxidizability (Porter, 1984; Gardner, 1989; Cosgrove et al., 1987; Barclay, 1993).

Although the oxidation of lipids is complex, the rate of oxidation of PUFA in homogeneous solution is kinetically predictable (Gardner, 1989; Barclay, 1993). From reactions 1–4 we obtain

$$-d[O_2]/dt = k_p[L-H](R_i/2k_t)^{1/2} \quad (6)$$

The susceptibility of lipids to oxidation, or oxidizability, is derived from eq 6:

$$\text{oxidizability} = k_p/(2k_t)^{1/2} \quad (7)$$

In studies using homogeneous solutions of purified lipids, a linear correlation was found between the number of *bis*-allylic methylene positions and the oxidizability of the lipids (Cosgrove et al., 1987). Thus, k_p varies linearly with the number of *bis*-allylic methylene positions; eq 7 can then be written as

$$\text{oxidizability} = \text{MBI} \times k_p'/(2k_t)^{1/2} \quad (8)$$

where MBI (methylene bridge index) represents the number of methylene bridge positions in the PUFA and k_p' is the propagation rate constant per methylene bridge position.

The concept of oxidizability is routinely applied to homogeneous lipid solutions; however, there has been no quantitative examination of its applicability to cellular systems. Previous studies with liposomes (Montfoort et al., 1987; Mowri et al., 1984; McLean & Hagaman, 1992; Barclay, 1993), subcellular fractions (Canuto et al., 1991), cells (Fraga et al., 1990; Hart et al., 1991; Burns & Wagner, 1991; North et al., 1992; Wagner et al., 1992, 1993), or tissues (Garrido et al., 1989; Wills, 1985) have not always been quantitative in that precise determination of the double bond content of these systems was not reported. However, the general trend observed is consistent with an increase in susceptibility to peroxidation as the degree of unsaturation increases.

In this study we have combined oxygen uptake and electron paramagnetic resonance spin trapping to examine the lipid oxidizability of intact cells. We have systematically altered the unsaturation of L1210 cellular lipids by supplementing their growth medium with various polyunsaturated fatty acids. We then did a complete lipid analysis of the cells to determine the actual level of lipid unsaturation. Using these data, we determined an exact MBI for a given cell preparation. The cells were subjected to an oxidative stress to determine their apparent oxidizability. We report here that the apparent oxidizability of cellular lipids correlates *exponentially* with the number of *bis*-allylic methylene positions in the cellular fatty acids.

MATERIALS AND METHODS

Cell Culture and Lipid Modification. L1210 murine leukemia stock cultures were maintained in RPMI 1640 media with 5% heat-inactivated fetal bovine serum (FBS). Cellular fatty acids were modified by growing cells for 48 h in media that had been supplemented with 32 μ M of one of the following fatty acids (*cis* form from Nu Chek Prep, Inc., Elysian, MN): oleic acid, 18:1 ω 9; linoleic acid, 18:2 ω 6; linolenic acid, 18:3 ω 3; γ -linolenic acid, 18:3 ω 6; arachidonic acid, 20:4 ω 6; eicosapentaenoic acid, 20:5 ω 3; docosatetraenoic acid, 22:4 ω 6; docosahexaenoic acid, 22:6 ω 3 (Burns & Wagner, 1991); Guffy et al., 1984, 1982; Burns & North, 1986; Burns et al., 1988). Fatty acids are abbreviated as number of carbon atoms: number

of double bonds; ω refers to the position of the first double bond from the methyl terminus. This concentration of fatty acid has no effect on cell viability (Guffy et al., 1984). Following fatty acid modification, the cells were washed twice by centrifugation (300g) through 0.9% NaCl. Cell densities were adjusted to 5×10^6 cells/mL in 0.9% NaCl for EPR studies.

There was no difference in cellular vitamin E content in lipid-modified cells compared to unmodified cells.²

Calculation of the Methylene Bridge Index (MBI) and the Double Bond Index (DBI). Fatty acid composition of cells was determined using gas chromatography (Burns & Wagner, 1991; Guffy et al., 1984; Burns et al., 1979). The mean number of *bis*-allylic methylene bridge positions per fatty acid contained in cells, expressed as MBI, was calculated by multiplying the number of *bis*-allylic methylene bridge positions contained in each fatty acid methyl ester species by its respective mole fraction and summed for all fatty acids present. The number of double bonds, DBI, was calculated similarly.

EPR Studies during Lipid Peroxidation. Lipid peroxidation experiments were carried out in 0.9% NaCl with no added lipids to ensure that oxidations occurred in the cells, essentially as described previously (Wagner et al., 1992, 1993). Briefly, reagents were added to cell suspensions in respective order to achieve 50 mM α -(1-oxo-4-pyridyl)-*N*-*tert*-butyl nitron (POBN), 100 μ M L-ascorbic acid, and 20 μ M FeSO₄·7H₂O. Consecutive EPR spectra were taken at 23.5-s intervals with a Bruker ESP 300 spectrometer at room temperature. After the ninth scan (218 s), 40 μ M 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH₃, edelfosine, Medmark GmbH, Grünwald, Germany, kindly supplied by Dr. R. Nordström) in ethanol was added as a prooxidant (Wagner et al., 1993). EPR settings were 9.79 GHz, 100-kHz modulation frequency, 40-mW nominal microwave power, 0.63-G modulation amplitude, 328-ms time constant, scanning at 60 G/21 s, and receiver gain 2.5×10^5 . Quantitative estimates of POBN adduct concentrations in the over 2500 EPR spectra collected were determined using 3-carboxyproxyl (Aldrich Chemical Co., Milwaukee, WI) as a standard (Buettner, 1990).

Oxygen Uptake. Oxygen uptake was monitored with a YSI Model 53 biological oxygen monitor (Yellow Springs, OH). Incubations were identical to those used in the EPR studies and were run in a parallel manner.

RESULTS

Fatty Acid Modification. L1210 cells that had been grown in media supplemented with various fatty acids were considerably modified (Table 1, individual acids). In most cases, enrichment was in the specific fatty acid added to the growth media, and it varied from 5% to 62%. The enrichment was >18% for all but two fatty acids; 18:3 ω 6 was elongated to 20:3 (24%) and 20:5 was elongated to 22:5 (18%). There was also limited elongation of 18:2 (7%) and 20:4 (12%).

This enrichment and metabolism had a major effect on overall unsaturation of cellular fatty acids (Table 1, classes of fatty acids and indices). The mole percent of polyunsaturated fatty acids ranged from 9% to 64%. Most importantly, the DBI ranged from 1.04 to 2.12 and the MBI from 0.27 to 1.5. This wide range of lipid unsaturation provided a powerful model to experimentally test its effect on lipid-derived radical generation and related peroxidative events.

² E. E. Kelley, G. R. Buettner, and C. P. Burns, unpublished observations (manuscript in preparation).

Table 1: Fatty Acid Composition of L1210 Cells (in mol %)^a

fatty acid	fatty acid added to media								
	unmodified	18:1 ω 9	18:2 ω 6	18:3 ω 3	18:3 ω 6	20:4 ω 6	20:5 ω 3	22:4 ω 6	22:6 ω 3
individual acids^b									
14:0	1.9 $\pm$ 0.1	2.3 $\pm$ 0.8	3.6 $\pm$ 1.9	2.6 $\pm$ 1.1	3.4 $\pm$ 0.6	2.0 $\pm$ 0.3	2.5 $\pm$ 0.4	12.2 $\pm$ 4.2	4.0 $\pm$ 0.7
16:0	16.5 $\pm$ 1.2	10.3 $\pm$ 0.7	9.4 $\pm$ 0.5	11.5 $\pm$ 1.5	17.8 $\pm$ 0.8	16.9 $\pm$ 1.6	16.6 $\pm$ 1.3	17.2 $\pm$ 0.9	20.7 $\pm$ 0.7
16:1	4.0 $\pm$ 0.3	2.2 $\pm$ 0.3	0.9 $\pm$ 0.1	2.3 $\pm$ 0.4	1.4 $\pm$ 0.1	2.0 $\pm$ 0.1	2.0 $\pm$ 0.2	1.7 $\pm$ 0.1	1.6 $\pm$ 0.2
18:0	22.4 $\pm$ 0.5	9.3 $\pm$ 0.6	11.8 $\pm$ 2.3	13.0 $\pm$ 1.2	16.3 $\pm$ 1.2	14.8 $\pm$ 1.1	17.5 $\pm$ 1.5	14.2 $\pm$ 0.7	19.9 $\pm$ 0.3
18:1 ω 9	34.4 $\pm$ 2.8	62.0 $\pm$ 1.7	8.2 $\pm$ 0.9	12.7 $\pm$ 1.2	18.0 $\pm$ 2.4	21.5 $\pm$ 3.0	18.4 $\pm$ 2.4	17.1 $\pm$ 2.3	19.7 $\pm$ 1.2
18:2 ω 6	2.8 $\pm$ 0.5	1.3 $\pm$ 0.1	50.4 $\pm$ 3.3	1.7 $\pm$ 0.1	1.4 $\pm$ 0.2	1.3 $\pm$ 0.1	2.0 $\pm$ 0.3	1.6 $\pm$ 0.1	1.3 $\pm$ 0.1
18:3 ω 3	1.1 $\pm$ 0.7	0.0	0.1 $\pm$ 0.1	39.4 $\pm$ 7.1	0.7 $\pm$ 0.1	0.6 $\pm$ 0.3	1.0 $\pm$ 0.3	0.2 $\pm$ 0.1	1.0 $\pm$ 0.1
18:3 ω 6	0.2 $\pm$ 0.1	0.0	0.0	0.0	4.6 $\pm$ 1.6	0.1 $\pm$ <0.1	0.1 $\pm$ <0.1	0.0	0.0
20:2 ω 6	0.1 $\pm$ <0.1	0.3 $\pm$ 0.2	6.7 $\pm$ 0.6	0.0	0.1 $\pm$ 0.1	0.1 $\pm$ <0.1	0.1 $\pm$ 0.1	0.4 $\pm$ 0.2	0.3 $\pm$ 0.1
20:3 ω 6	1.2 $\pm$ 0.1	0.7 $\pm$ 0.2	1.0 $\pm$ 0.1	0.7 $\pm$ 0.2	24.1 $\pm$ 2.8	1.2 $\pm$ 0.1	1.5 $\pm$ 0.2	1.3 $\pm$ 0.3	1.3 $\pm$ 0.4
20:4 ω 6	6.2 $\pm$ 1.2	3.0 $\pm$ 0.1	1.6 $\pm$ 0.2	7.9 $\pm$ 0.7	5.1 $\pm$ 0.5	22.5 $\pm$ 0.5	4.4 $\pm$ 0.3	7.4 $\pm$ 0.8	2.3 $\pm$ 0.3
20:5 ω 3	0.9 $\pm$ 0.4	0.4 $\pm$ 0.2	1.6 $\pm$ 0.5	0.4 $\pm$ 0.1	0.1 $\pm$ <0.1	<0.1 $\pm$ <0.1	11.3 $\pm$ 1.0	1.6 $\pm$ 0.7	0.9 $\pm$ 0.1
22:4 ω 6	1.7 $\pm$ 0.9	1.7 $\pm$ 0.5	1.0 $\pm$ 0.3	2.6 $\pm$ 1.4	1.2 $\pm$ 0.1	12.5 $\pm$ 0.8	0.7 $\pm$ 0.1	17.6 $\pm$ 2.1	0.6 $\pm$ <0.1
22:5 ω 3	1.4 $\pm$ 0.1	1.0 $\pm$ 0.3	1.0 $\pm$ 0.1	1.2 $\pm$ 0.3	1.0 $\pm$ 0.1	1.1 $\pm$ 0.1	18.4 $\pm$ 2.4	2.8 $\pm$ 0.5	1.0 $\pm$ 0.1
22:6 ω 3	1.7 $\pm$ 0.1	0.7 $\pm$ 0.2	0.3 $\pm$ 0.1	1.2 $\pm$ 0.3	2.4 $\pm$ 1.0	2.0 $\pm$ 0.3	1.7 $\pm$ 0.3	1.6 $\pm$ 0.7	23.3 $\pm$ 1.1
others	3.5	4.8	2.4	2.8	2.4	1.3	1.8	3.1	2.1
classes of fatty acids									
% PU ^c	17.3 $\pm$ 1.3	9.1 $\pm$ 0.6	63.6 $\pm$ 3.6	55.2 $\pm$ 5.0	40.7 $\pm$ 4.5	41.5 $\pm$ 0.8	41.0 $\pm$ 4.2	35.1 $\pm$ 1.7	31.8 $\pm$ 1.8
% MU	41.4 $\pm$ 2.9	68.7 $\pm$ 1.8	10.8 $\pm$ 0.5	17.3 $\pm$ 2.1	20.4 $\pm$ 2.7	24.4 $\pm$ 3.1	22.1 $\pm$ 2.5	20.4 $\pm$ 1.9	23.3 $\pm$ 1.5
% SAT	41.3 $\pm$ 1.6	22.2 $\pm$ 2.2	25.6 $\pm$ 3.9	27.5 $\pm$ 2.9	38.9 $\pm$ 2.1	34.1 $\pm$ 2.4	36.9 $\pm$ 3.0	44.5 $\pm$ 3.2	44.9 $\pm$ 0.3
% ω 3	5.1 $\pm$ 1.2	2.1 $\pm$ 0.4	2.9 $\pm$ 0.4	42.2 $\pm$ 6.5	4.2 $\pm$ 1.0	3.8 $\pm$ 0.2	32.3 $\pm$ 3.5	6.8 $\pm$ 1.1	26.1 $\pm$ 1.4
% ω 6	12.2 $\pm$ 1.8	7.0 $\pm$ 0.2	60.7 $\pm$ 4.0	12.9 $\pm$ 1.7	36.4 $\pm$ 3.9	37.7 $\pm$ 0.8	8.8 $\pm$ 0.8	28.3 $\pm$ 2.7	5.8 $\pm$ 0.5
indices									
DBI ^c	1.08 $\pm$ 0.02	1.04 $\pm$ 0.05	1.53 $\pm$ 0.07	1.99 $\pm$ 0.10	1.57 $\pm$ 0.14	1.91 $\pm$ 0.01	2.12 $\pm$ 0.18	1.63 $\pm$ 0.08	1.94 $\pm$ 0.08
MBI	0.49 $\pm$ 0.04	0.27 $\pm$ 0.03	0.79 $\pm$ 0.03	1.26 $\pm$ 0.07	0.96 $\pm$ 0.12	1.25 $\pm$ 0.02	1.50 $\pm$ 0.15	1.07 $\pm$ 0.04	1.38 $\pm$ 0.07

^a L1210 cells were grown for 48 h in media supplemented at 32 μ M with the fatty acid designated in the column headings. Cells were washed and extracted with CHCl₃-CH₃OH, 2:1 (v/v). After alkaline hydrolysis, fatty acids in the saponifiable fraction were methylated and the methyl esters separated by gas-liquid chromatography. Mole percents of various fatty acids were calculated on the basis of weight percents from integrated peak areas on chromatograms and respective molecular weights of fatty acid methyl esters. ^b Expressed as mole percent of total fatty acids. Fatty acids are designated as number of carbon atoms:number of double bonds. Values are the mean $\pm$ SE of three independent experimental sample determinations. ^c Abbreviations: PU, polyunsaturated fatty acids; MU, monosaturated fatty acids; SAT, saturated fatty acids; DBI, double bond index which is the mean number of double bonds per fatty acid; MBI, methylene bridge index which is the mean number of *bis*-allylic positions per fatty acid.

POBN Lipid Radical Adduct Formation. Lipid-derived free radicals were detected by EPR spin trapping when L1210 cells were subjected to iron-ascorbate/ether lipid-induced oxidative stress (Figure 1, inset). The POBN spin adduct EPR signal observed has hyperfine splitting parameters ($a^N = 15.61$ G; $a^H = 2.64$ G) identical to those of the carbon-centered lipid-derived spin adducts previously observed from intact cells subjected to free radical-mediated oxidative stress (North et al., 1992; Wagner et al., 1993; Buettner et al., 1993). By monitoring the POBN/L[•] EPR signal intensity changes with time, we were able to study the effect of cell lipid composition on the rate of peroxidation (Figure 1). The initial rate of cellular lipid peroxidation is highly variable. Unmodified and 18:1-modified cells had the lowest rates of peroxidation, while 20:5- and 22:6-modified cells had the highest rates. In summary, the greater the degree of unsaturation in the modifying lipid, the more easily oxidizable the cellular lipids.

In homogeneous solution, the rate of lipid peroxidation is linearly correlated to the number of *bis*-allylic positions in the lipids (Cosgrove et al., 1987). Using dilinoleoylphosphatidylcholine/dimyristoylphosphatidylcholine mixed liposomes, Barclay (1993) found that aggregation of lipid chains into bilayers does not change their oxidizability. In our study the rate of POBN/L[•] formation from cells exposed to free radical oxidative stress is related to the oxidizability of the cellular lipids. Much to our surprise, in cells these rates increased *exponentially* as the MBI increased in the various lipid-modified cell preparations (Figure 2) ($r = 0.962$ and $p = 3.4 \times 10^{-5}$ for a logarithmic correlation versus $r = 0.812$ and $p = 7.8 \times 10^{-3}$ for an arithmetic, linear correlation). The maximum level of POBN/L[•] also increased with the MBI of the various cell preparations (Figure 3) ($r = 0.948$ and $p =$

9.9×10^{-5} for a logarithmic correlation versus $r = 0.944$ and $p = 1.3 \times 10^{-4}$ for an arithmetic, linear correlation).

Cellular Site of Lipid Radical Generation. There is cell injury during the incubations as a result of the oxidative stress. For example, 22:6-modified cell counts decreased to $93 \pm 2\%$, $72 \pm 4\%$, $66 \pm 1\%$, and $55 \pm 3\%$ at 60, 300, 900, and 1800 s. This is undoubtedly due to peroxidation of cellular membranes and could result in the release of lipids into the media. Therefore, the lipid radical that we detected could be due to lipid peroxidation occurring in the media rather than within cells. On the basis of evidence from two experiments designed to examine this question, we believe that the radical is not originating from lipids released into the media. In the first experiment, 22:6-enriched L1210 cells were subjected to oxidative stress, and then the media and cells were separated by centrifugation at 100000g. The lipid radical generated by the media with additional exposure to the oxidative stress was limited and reached a maximum intensity of only 2-fold above the initial baseline (compare to Figure 1), and most time points were less. When the cells were not exposed to oxidative stress prior to centrifugation, there was no lipid radical detected in the media, indicating that it is unlikely that the reaction is initiated in the media. In the second experiment, 22:6-enriched L1210 cells were grown for 24 h in 100 μ M vitamin E acetate and then exposed to the oxidative stress. The lipid-soluble vitamin E, which localizes in membranes, inhibited lipid-derived radical production by 65%, 93%, 82%, and 77% at 60, 300, 600 and 900 s; this compares to only 0%, 11%, 18%, and 12% inhibition by 100 μ M Trolox (CAS Registry No. 53188-07-1 supplied by Aldrich Chemical Co.) added just prior to the oxidative stress. Trolox is a vitamin E analogue that is water soluble and remains in the media. Taken together, these data suggest that the vast majority of the radical we

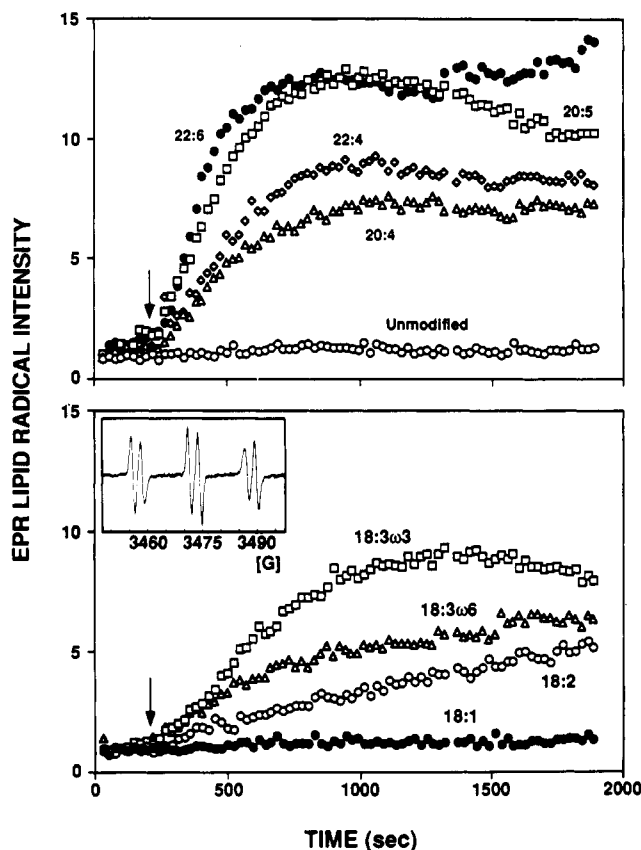


FIGURE 1: Time course of lipid peroxidation as measured by POBN/ $L^{\bullet}$ formation using L1210 cells enriched with various fatty acids. Fatty acid-enriched cells, 5×10^6 cells/mL, were monitored by EPR after initiation of peroxidation with $20 \mu\text{M Fe}^{2+}$ and $100 \mu\text{M}$ ascorbic acid. After initiation (218 s), $40 \mu\text{M MET-18-OCH}_3$ was added (arrow). Values are the mean from three independent experiments for each of the fatty acid-enriched cell types. Maximum levels of POBN/ $L^{\bullet}$ adducts were $1.7\text{--}1.9 \mu\text{M}$ for 20:5 and 22:6, compared to $\approx 0.2 \mu\text{M}$ in unmodified and 18:1-enriched cells. The inset shows a typical POBN/ $L^{\bullet}$ EPR spectrum: $a^N = 15.61 \text{ G}$; $a^H = 2.64 \text{ G}$. At early time points the ascorbate radical doublet, $a^H = 1.8 \text{ G}$, can also be observed. This particular spectrum is from the $\approx 600\text{-s}$ time point in an experiment with 20:5-enriched cells.

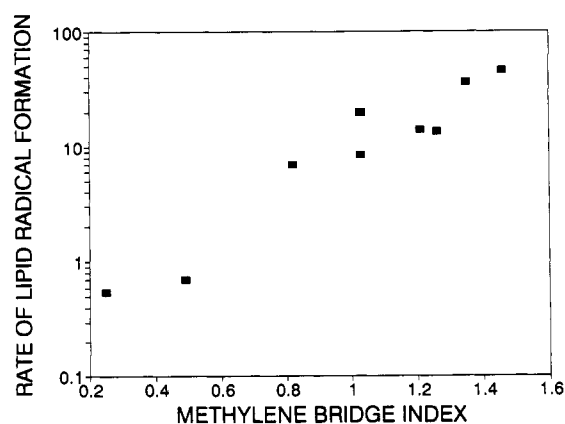


FIGURE 2: Relationship of rate of formation of POBN/ $L^{\bullet}$ versus *bis*-allylic methylene bridge positions of cells (MBI). Presented are the median values for each of the nine different fatty acid-modified cell preparations from triplicate determinations: $r = 0.962$; $p = 3.4 \times 10^{-5}$. The rates were determined from the regions of the curves of Figure 1 that best represented initial rates. Regression analysis was done on an appropriate subset of data from the 250–1000-s range.

observed as POBN/ $L^{\bullet}$ is generated from cells and not from fatty acids or complex lipids released into the media.

Oxygen Consumption. Monitoring by EPR the formation of POBN/ $L^{\bullet}$ is a means of following product production in

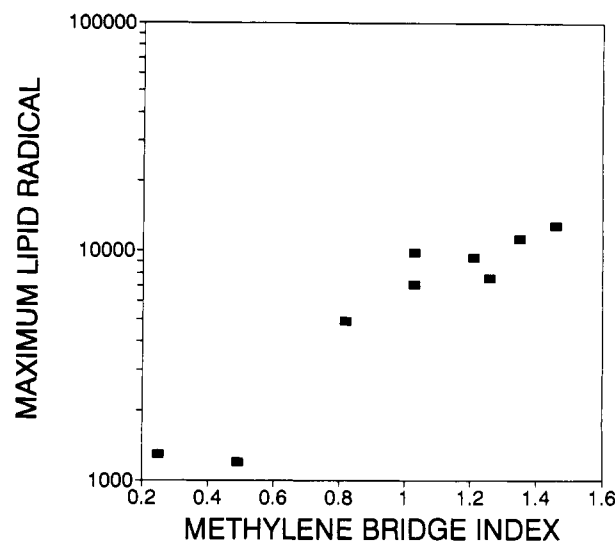


FIGURE 3: Maximum POBN/ $L^{\bullet}$ EPR intensity. The maximum values of POBN/ $L^{\bullet}$ intensity values from Figure 1 are shown versus the MBI. A logarithmic correlation is observed: $r = 0.948$; $p = 9.9 \times 10^{-5}$. The arithmetic correlation on nonlogarithmic scales is similar: $r = 0.944$; $p = 1.3 \times 10^{-4}$.

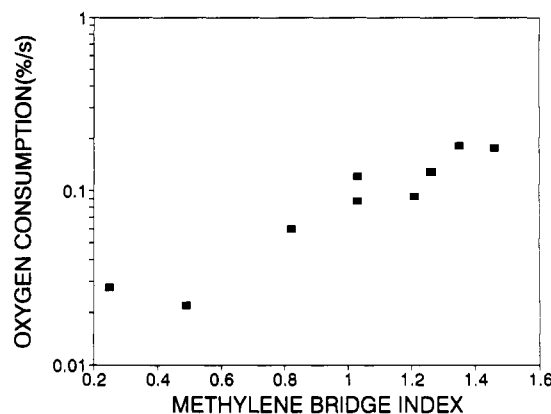


FIGURE 4: Relationship of rate of oxygen consumption, expressed as percent oxygen lost per second, and number of *bis*-allylic methylene hydrogen positions. Values are the median from three independent experiments for cells enriched with each of the nine different fatty acids: $r = 0.950$; $p = 8.7 \times 10^{-5}$. These rates were determined in the same manner as described in Figure 2.

real time during lipid peroxidation. To ensure that our observation that cellular lipid oxidizability varies exponentially versus MBI was not an artifact of this method, we simultaneously monitored oxygen uptake; oxygen uptake examines reactant disappearance. As with POBN/ $L^{\bullet}$, a positive logarithmic correlation was found between the rate of oxygen uptake (oxidizability) and MBI (Figure 4) ($r = 0.950$ and $p = 8.7 \times 10^{-5}$ for a logarithmic correlation versus $r = 0.913$ and $p = 5.8 \times 10^{-4}$ for a linear correlation).

DISCUSSION

From our studies it is clear that cellular susceptibility to lipid peroxidation *in vitro* is dependent upon the degree of polyunsaturation of cellular lipids. We have demonstrated quantitatively that the number of *bis*-allylic positions from which hydrogen can be abstracted by free radical processes is the major determinant for the oxidizability of cellular lipids, confirming theoretical considerations, *vide supra*. In our studies, hydrogen abstraction from carbons not located between double bonds has a minimal contribution to free radical generation and is consistent with observations in pure lipid systems where it contributes $<1\%$ of peroxidation reactions

(Gardner, 1989). The low levels of POBN/L[•] formation with cells enriched with 18:1, which has no double allylic positions, reveal the limited influence that single, isolated double bonds *per se* have on cellular lipid peroxidation events. For comparison, cells enriched with 18:2, having only a single *bis*-allylic position, produce substantially more lipid radical.

The influence of fatty acid chain length was difficult to assess since the level of unsaturation of the fatty acids used in our model increased as the chain length increased. There was a slightly higher peak radical intensity (Figure 1) and rate of radical generation (47% greater) from cells enriched with 22:4 as compared to 20:4, in spite of a slightly lower MBI in 22:4 cells. Likewise, the role of ω -family is indefinite. Others have reported differences in the preferential peroxidation of some double bond positions, especially double bonds located in the terminal positions of some fatty acids (Porter, 1984). In our studies, the peak level and rate of generation for 18:3 ω 3 were somewhat higher than for 18:3 ω 6; however, the MBI for 18:3 ω 3 was higher.

Taken together from both reactant disappearance (O₂ uptake) and product formation (POBN/L[•]), we conclude that the apparent cellular lipid oxidizability increases *exponentially* as the PUFA content of cells increases. This contrasts with results from homogeneous solutions of lipids where a linear correlation is well established (Cosgrove et al., 1987). One possibility to explain this apparent exponential increase in oxidizability is a clustering of lipids within cell membranes. This clustering would result in an increase in the rate of peroxidation events analogous to that observed when lipid oxidation processes were studied in solutions where the lipid concentration is varied from below to above the critical micelle concentration (Patterson, 1981).³ The closer packing of the unsaturated lipids in a structured environment may result in our observed increase in the apparent oxidizability of these lipids.

Previous work in several laboratories, including our own, has shown that modification of peroxidizable lipids in cell membranes correlates with changes in susceptibility to oxidant-mediated cytotoxicity (Guffy et al., 1984; Spitz et al., 1992; Hart et al., 1991). We have clearly demonstrated previously that increasing the proportion of unsaturated lipids in cell membranes increases lipid radical formation and decreases cell survival when subjected to an oxidative stress (Wagner et al., 1992, 1993; Buettner et al., 1993). Our quantitative observations here on the influence of *bis*-allylic positions on cellular lipid peroxidation provide a sound basis for understanding the role of polyunsaturation in cellular, lipid peroxidation events.

REFERENCES

- Barclay, L. R. C. (1993) *Can. J. Chem.* 71, 1–16.
- Buettner, G. R. (1990) *Free Radical Res. Commun.* 10, 11–15.
- Buettner, G. R. (1993) *Arch. Biochem. Biophys.* 300, 535–543.
- Buettner, G. R., Kelley, E. E., & Burns, C. P. (1993) *Cancer Res.* 53, 3670–3673.
- Burns, C. P., & North, J. A. (1986) *Biochim. Biophys. Acta* 888, 10–17.
- Burns, C. P., & Wagner, B. A. (1991) *J. Lipid Res.* 32, 79–87.
- Burns, C. P., Luttenegger, D. G., Dudley, D. T., Buettner, G. R., & Spector, A. A. (1979) *Cancer Res.* 39, 1726–1732.
- Burns, C. P., North, J. A., Mossman, C. J., & Ingraham, L. M. (1988) *Lipids* 23, 615–618.
- Canuto, R. A., Muzio, G., Biocca, M. E., & Dianzani, M. U. (1991) *Cancer Res.* 51, 4603–4608.
- Cosgrove, J. D., Church, D. F., & Pryor, W. A. (1987) *Lipids* 22, 299–304.
- Fraga, C. G., Tappel, A. L., Leibovitz, B. E., Kuypers, F., Chiu, D., Iacono, J. M., & Kelley, D. S. (1990) *Lipids* 25, 111–114.
- Gardner, H. W. (1989) *Free Radical Biol. Med.* 7, 65–86.
- Garrido, A., Garrido, F., Guerra, R., & Valenzuela, A. (1989) *Lipids* 24, 833–835.
- Guffy, M. M., Rosenberger, J. A., Simon, I., & Burns, C. P. (1982) *Cancer Res.* 42, 3625–3630.
- Guffy, M. M., North, J. A., & Burns, C. P. (1984) *Cancer Res.* 44, 1863–1866.
- Hart, C. M., Tolson, J. K., & Block, E. R. (1991) *Am. J. Physiol.* 260, L481–L488.
- Hasegawa, K., & Patterson, L. K. (1978) *Photochem. Photobiol.* 28, 817–823.
- Koppenol, W. H. (1990) *FEBS Lett.* 264, 165–167.
- McLean, L. R., & Hagaman, K. A. (1992) *Free Radical Biol. Med.* 12, 113–119.
- Montfoort, A., Bezstarosti, K., Groh, M. M. J., & Metsä-Ketelä, T. J. A. (1987) *Biochem. Int.* 15, 525–543.
- Mowri, H., Nojima, S., & Inoue, K. (1984) *J. Biochem.* 95, 551–558.
- North, J. A., Spector, A. A., & Buettner, G. R. (1992) *J. Biol. Chem.* 267, 5743–5746.
- Patterson, L. K. (1981) in *Oxygen and Oxy-Radicals in Chemistry and Biology* (Rodgers, M. A. J., & Powers, E. L., Eds.) pp 89–95, Academic Press, New York.
- Porter, N. A. (1984) *Methods Enzymol.* 105, 273–282.
- Spitz, D. R., Kinter, M. T., Kehrer, J. P., & Roberts, R. J. (1992) *Pediatr. Res.* 32, 366–372.
- Wagner, B. A., Buettner, G. R., & Burns, C. P. (1992) *Cancer Res.* 52, 6045–6051.
- Wagner, B. A., Buettner, G. R., & Burns, C. P. (1993) *Cancer Res.* 53, 711–713.
- Wills, E. D. (1985) in *Oxidative Stress* (Sies, H., Ed.) pp 197–218, Academic Press, London.

³ J. A. North and G. R. Buettner, unpublished observations.