

## Methods for estimating lipid peroxidation: An analysis of merits and demerits

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*Received 5 May 2003; revised 24 July 2003*

Among the cellular molecules, lipids that contain unsaturated fatty acids with more than one double bond are particularly susceptible to action of free radicals. The resulting reaction, known as lipid peroxidation, disrupts biological membranes and is thereby highly deleterious to their structure and function. Lipid peroxidation is being studied extensively in relation to disease, modulation by antioxidants and other contexts. A large number of by-products are formed during this process. These can be measured by different assays. The most common method used is the estimation of aldehydic products by their ability to react with thiobarbituric acid (TBA) that yield 'thiobarbituric acid reactive substances' (TBARS), which can be easily measured by spectrophotometry. Though this assay is sensitive and widely used, it is not specific and TBA reacts with a number of components present in biological samples. Hence caution should be used while employing this method. Wherever possible this assay should be combined with other assays for lipid peroxidation. Such methods are measurement of conjugated dienes, lipid hydroperoxides, individual aldehydes, exhaled gases like pentane, isoprostanes, etc. The modern methods also involve newer techniques involving HPLC, spectrofluorimetry, mass spectrometry, chemiluminescence etc. These and other modern methods are more specific and can be applied to measure lipid peroxidation. There are certain restraints, in terms of high cost and certain artifacts, and these should be considered while selecting the method for estimation. This review analyses the merits and demerits of various assays to measure lipid peroxidation.

**Keywords:** Lipid peroxidation, thiobarbituric acid reactive substances, malondialdehyde, polyunsaturated fatty acid, conjugated dienes, lipid hydroperoxides, estimation

### Introduction

Lipid peroxidation (LP) can be defined as the oxidative deterioration of lipids containing a number of carbon-carbon double bonds<sup>1</sup>. A large number of toxic by-products are formed during LP. These have effects at a site away from area of their generation. Hence they behave as toxic 'second messengers'. Membrane lipids are particularly susceptible to LP. Since membranes form the basis of many cellular

organelles like mitochondria, plasma membranes, endoplasmic reticulum, lysosomes, peroxisomes etc. the damage caused by LP is highly detrimental to the functioning of the cell and its survival<sup>2</sup>.

Presence of polyunsaturated fatty acids (PUFAs) in the phospholipids of the bilayer of biological membranes is the basis of their critical feature of fluidity. Since LP attacks the components that impart these properties, it affects the biophysical properties of membranes. LP decreases the membrane fluidity, changes the phase properties of the membranes and decreases electrical resistance. Also, cross-linking of membrane components restricts mobility of membrane proteins<sup>1,3</sup>. Peroxidative attack on PUFAs of a biological membrane will compromise one of its most important functions: its ability to act as barrier. Hence, LP causes lysosomes to have a decreased 'latency' i.e., they become fragile or simply 'leaky'. Similarly, the leakage of cytosolic enzymes from whole cells e.g. peroxidative attack on the plasma membrane of hepatocytes causes extensive damage such that molecules as large as enzymes are able to leak out<sup>1</sup>.

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**Abbreviations used:**  $\alpha$ -TOH,  $\alpha$ -tocopherol;  $\cdot$ OH, hydroxyl radical; 4-HNE, 4-hydroxynonenal; AAPH, 2,2'-azobis (amidinopropane) hydrochloride; BHT, butylated hydroxytoluene; ELISA, Enzyme-linked sorbent assay; FID, flame ionization detector; GC-MS, gas chromatography-mass spectrometry; GSH, glutathione; HCFA, hydrocarbon free air; HPLC, high performance (pressure) liquid chromatography; L $\cdot$ , lipid radical; LDL, low density lipoprotein; LH, unsaturated lipid; LO $\cdot$ , lipoxy radical; LOO $\cdot$ , lipid peroxy radical; LOOH, lipid hydroperoxide; LP, lipid peroxidation; MDA, malondialdehyde; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TPP, triphenylphosphine

Peroxidation is well known to decrease activities of enzymes associated with membranes. The most extensively studied examples are enzymes of the endoplasmic reticulum, glucose-6-phosphatase and cytochrome P450<sup>1,3-5</sup>. Mitochondrion and Golgi apparatus are also susceptible to LP. Membrane LP can result in inactivation of membrane pumps responsible for maintaining ionic homeostasis. Some enzyme activities can also be stimulated by LP e.g., increase in activity of phospholipase A<sub>2</sub> in membranes subjected to oxidative stress, to remove and replace toxic LP products in the membrane<sup>2,6-8</sup>.

Lipid peroxidation has been implicated in the pathogenesis of a number of diseases and clinical conditions. These include premature birth disorders, diabetes, adult respiratory distress syndrome, aspects of shock, Parkinson disease, Alzheimer disease, various chronic inflammatory conditions, ischemia-reperfusion mediated injury to organs including heart, brain, and intestine, atherosclerosis, organ injury associated with shock and inflammation, fibrosis and cancer, preeclampsia and eclampsia, inflammatory liver injury, type 1 diabetes, anthracycline-induced cardiotoxicity, silicosis and pneumoconiosis<sup>3,7,9-11</sup>. Experimental and clinical evidence suggests that aldehyde products of LP can also act as bioactive molecules in physiological and pathological conditions. These compounds can effect and modulate, at very low and non-toxic concentrations, several cell functions including signal transduction, gene expression, cell proliferation and more generally the response of target cells<sup>12</sup>.

Many of the products of LP are not overtly toxic or are minor products. Of major toxicological interest are malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and various 2-alkenals. A range of alterations is known to occur upon exposure of DNA to lipid hydroperoxide (LOOH). Incubation of plasmid DNA with auto-oxidized unsaturated fatty acids (linoleic or arachidonic), results in extensive single and double strand breaks. Such strand breaks have also been detected in human lymphocytes and fibroblasts after treatment with LOOH. Exposure of calf thymus to LOOH damaged its DNA (C-8 hydroxylation of guanine residues). A spectrum of SupF mutations was produced upon error prone replication of p2189 plasmids, following treatment with autoxidized rat microsomal lipids<sup>13-15</sup>.

### Products of lipid peroxidation

Oxygen-dependent deterioration of lipids, known as rancidity, has been noticed since antiquity as a

major problem in the storage of oils. It was also found useful as far back as the 15th century in preparing oil paints and printing inks. The same oxidation process also occurs in the case of natural products such as fats, oils, dressings or margarines and also chemical and industrial products, such as inks, paints, resins, varnishes or lacquers. Much information concerning the mechanism of the auto-oxidation of lipid compounds has been obtained by the study of the oxidation of simple non-fatty products such as cyclohexene. Since the early 1960's, our understanding of the oxidation of unsaturated lipids has advanced considerably as a result of the application of new analytical tools. Several research groups initiated detailed studies on the products of polyunsaturated fatty acids in the 70's to reveal more complex aspects of LP. With the help of HPLC, several hydroperoxide products could be separated after auto-oxidation of arachidonic acid<sup>16</sup>.

The first demonstration of free radical oxidation of membrane phospholipids was given in 1980<sup>17</sup>. LOOHs are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, esters and cholesterol. Their formation occurs in enzymatic or non-enzymatic reactions involving activated chemical species known as reactive oxygen species (ROS) that are responsible for toxic effects in the body via various types of tissue damage.

The major products, LOOHs, are fairly stable molecules at physiological temperatures. Transition metal complexes catalyzed their decomposition. A reduced iron compound can react with LOOH in a way similar to H<sub>2</sub>O<sub>2</sub> (Fenton reaction) causing fission of an O-O bond to form an alkoxyl radical. This radical also promotes the chain reaction of LP. MDA can be formed as the result of the fission of cyclic endoperoxides. With thiobarbituric acid (TBA), MDA readily forms an adduct. It can be measured spectrophotometrically by its characteristic pink colour<sup>1,3,13</sup>.

### Process of lipid peroxidation

The steps involved in iron-induced LP are described below and shown schematically in Fig. 1.

1.  $LH + X^{\bullet} \rightarrow L^{\bullet} + XH$
- 1a.  $LH + \text{active-Fe}^{2+} \rightarrow L^{\bullet} + \text{inactive-Fe}^{3+}$
2.  $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$
3.  $LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH$
4.  $LOOH + Fe^{2+} \rightarrow LO^{\bullet} + Fe^{3+}$
5.  $LOO^{\bullet} \rightarrow \text{MDA and nonenal}$

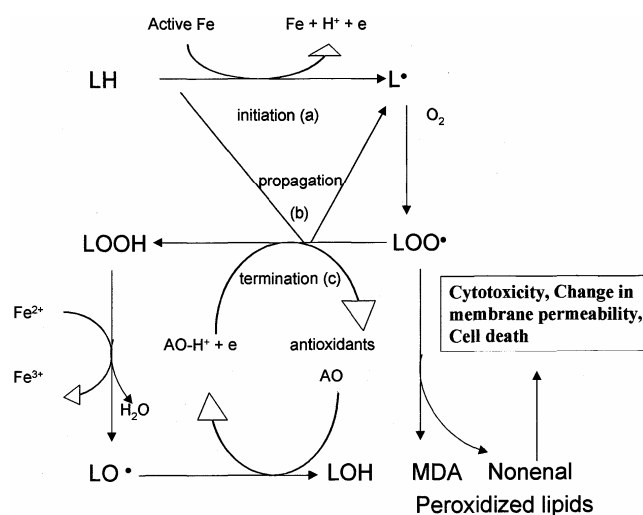
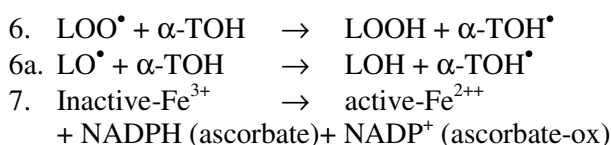


Fig. 1—Mechanisms involved in iron-induced lipid peroxidation



A peroxidative sequence is initiated by the attack of an unsaturated lipid (LH) by any species ( $\bullet\text{X}$  or active- $\text{Fe}^{2+}$ ) that abstracts hydrogen atom from a methylene group ( $-\text{CH}_2$ ). This leaves behind an unpaired electron on the carbon atom (reaction 1). Methylene groups adjacent to double bonds are particularly susceptible to attack, since their presence weakens the adjacent carbon-hydrogen bond<sup>1</sup>. The resultant carbon radical ( $\bullet\text{CH}$ ) is stabilized by molecular rearrangement to produce a conjugated diene. It can readily react with an oxygen molecule forming lipid peroxyl radical ( $\text{LOO}^\bullet$ ) (reaction 2). These radicals can abstract hydrogen atoms from other lipid molecules (LH) to become LOOH. When  $\text{L}^\bullet$  is formed from second LH (reaction 3), LP is propagated. LOOH can further be degraded by a Fenton-type reaction in presence of  $\text{Fe}^{2+}$  to another radical  $\text{LO}^\bullet$  (reaction 4).  $\text{LOO}^\bullet$  is unstable and breaks down to form various products including aldehydes, such as malondialdehyde (MDA) and 4-hydroxy-nonenal (reaction 5). MDA and related aldehydes are the most commonly estimated products of lipid peroxidation.

Peroxidation can be terminated by a number of reactions. The major one involves the reaction of (reaction 6) or  $\text{LO}^\bullet$  (reaction 6a) with antioxidants. Most effective of these is membrane-based  $\alpha$ -tocopherol ( $\alpha\text{-TOH}$ , vitamin E) forming more stable

tocopherol phenoxyl radical that can be 'recycled' by other cellular antioxidants, such as ascorbate (vitamin C) or glutathione (GSH)<sup>18</sup>. Oxidation of NADPH in enzyme systems, and of ascorbate in non-enzymic reaction regenerates the active- $\text{Fe}^{2+}$  (reaction 7).

### Problems faced in understanding lipid peroxidation process

The reactions in lipid peroxidation process are complicated and are not completely understood. One must know the roles of the many players. Some are listed below.

1. *What are the reduced iron and a reducing agent, ascorbate, doing in an oxidation process?* Certainly an active form of iron, some kind of an oxidant needed in the next step, is being produced. The primary oxidation relates to ascorbate oxidation, instinctively by oxygen. No one checked whether ascorbate is consumed, and if so stoichiometric with what other component.
2. *Where are the electrons of NADPH needed?* In the enzymic equivalent, NADPH replaces ascorbate and the microsomal membranes supply both the lipid substrate and the enzyme that uses the P450 system. In this case a ratio of 1: 4: 16 for MDA: NADPH:  $\text{O}_2$  is reported<sup>19</sup>. Indeed NADPH and oxygen are consumed far in excess of MDA. This implies that MDA is not the only product. Two electrons per mole of NADPH are available for reduction. It appears these are used for reducing iron to later become the suggested oxidant perferryl species. In that case  $\text{Fe}:\text{NADPH}$  ratio will be 2:1.
3. *Specificity of TBA reagent.* It is good to understand that the TBARS reaction is not that specific to MDA. Under the conditions employed any sugar can yield a pink colored product. Microsomes prepared in sucrose should not be used without giving them a wash in saline. Normally blanks should be checked.
4. *What is the primary oxidant of fatty acid?* It is the iron oxidant species that extracts the proton and electron. Some other metals are capable of substituting iron salts. Those interested must read the classic paper of Wills published a long time ago to get the right picture<sup>20</sup>. The critical step is the removal of one proton and one electron from a carbon in the middle of the fatty acid chain. This leaves behind a carbon radical. The most misleading of all is that  $\bullet\text{OH}$  initiates lipid peroxidation, which some authors use with little

thought given to its veracity. A radical produced, then what better than a  $\cdot\text{OH}$  to do this job of electron withdrawal. But, the best-known  $\cdot\text{OH}$  generator, a mixture of ferrous salt and  $\text{H}_2\text{O}_2$ , does not promote lipid peroxidation, nor do the many hydroxyl radical quenching agents stop it. Repeatedly many workers who know the field state this. Yet statements continue to appear in papers carrying this mistaken notion, unbelievably from some peers.

5. *What happens to the fatty acid?* After the iron oxidant steals the electron, what is left behind is a "hole" on a carbon of the fatty acid chain. This reactive carbon radical is immediately attacked by molecular oxygen to form a hydroperoxy radical ( $\text{LOO}\cdot$ ), with the oxygen atom becoming the radical species. Any number of reducing and phenolic compounds can reduce this to  $\text{LOOH}$  or to  $\text{LOH}$  after breakdown to  $\text{LO}\cdot$ . In either case, the chain is terminated. Many oxidized derivatives are the products of lipid peroxidation. Indeed this is why the process has come to be known as lipid peroxidation. Lipid oxidation is a more comprehensive name, but confuses with  $\beta$ -oxidation. These  $\beta$  reactions are not possible with the saturated or monounsaturated fatty acids. So they are stable and oils containing these become preferred cooking medium, albeit devoid of essential fatty acids. Introducing the second double bond enhances the rate of lipid peroxidation by 30-fold, and the third one by 100-fold<sup>21</sup>. These rate enhancements are achieved because of the ability of the chain to conjugate after peroxidation. The conjugated dienes are detected by absorbance at 233 nm (molar extinction for linoleate:  $27,400 \text{ M}^{-1} \text{ cm}^{-1}$ ). The values of peroxide and conjugated diene of the oxidized lipid correlate with that of oxygen consumed<sup>22</sup>. Then where is the much-touted chain reaction initiated by free radicals? This is based on paper chemistry, accepted without scrutiny. A good experiment on the chain reaction is yet to appear. If that was so, oxygen consumption should go on even after NADPH (or ascorbate?) was exhausted. But, why does it stop?

Many recent studies use simpler systems for inducing lipid peroxidation. In one such system the free radicals originate from the action of the 2,2'-azobis (amidinopropane) hydrochloride (AAPH), which has been successfully introduced as an initiator

of lipid peroxidation independently by Niki and Ingold<sup>23,24</sup>. The widely accepted mechanism for the thermal decomposition of AAPH provides carbon- and oxygen-centred radicals<sup>23-25</sup>. A recent study by Stocker *et al.* show that AAPH degradation induced erythrocytes lysis in oxygenated blood, but not in nitrogen saturated blood. In oxygenated blood, additional nitrones induced the formation of  $\text{Cl}(\text{NH}_2)_2\text{C}-(\text{Me})_2\text{CO}\cdot$  alkoxy radical adducts<sup>26</sup>.

### Methods for estimating lipid peroxidation

In recent years, a large number of assays have been developed for measuring MDA and other products of lipid peroxidation (Table 1). However, for the ease of operation and 'quick results' TBARS assay is still being followed in many laboratories, without realizing the artifacts and the limitations of the assay. One of the major objectives of this review is to cover these lacunae in the study of lipid peroxidation and its application to the studies in relation to human health.

The products of LP that can be measured include conjugated dienes, lipid hydroperoxides, aldehydes e.g. 4-hydroxynonenal and MDA that react with TBA to form TBARS, isoprostanes, isofurans, exhaled gases such as pentane, lipid-protein adducts, lipid-DNA adducts etc<sup>27</sup>.

The progression of lipid peroxidation can be monitored by measuring products of LP such as conjugated dienes, lipid hydroperoxides, aldehydes, aldehyde-protein adducts, alkanes or the depletion of substrates like PUFAs or antioxidants, besides chemiluminescence. The measurement of several oxidation products enables assessment at different stages of the oxidative pathway, providing detailed information of this dynamic process. Until recently, TBARS test was probably the most utilised assay for measuring lipid peroxidation. Although this approach is still used in some parts of the food industry, for instance, in testing the oxidative susceptibility of cooking oils and can provide a valuable laboratory

Table 1—Some methodologies used for studying lipid peroxidation

Component	Method	References
TBARS	Spectrophotometric, Spectrofluorimetric	28-30
Lipid hydroperoxides	Spectrophotometric	31,32
Conjugated dienes	Spectrophotometric	31,33
4-Hydroxynonenal	HPLC	34
Isoprostanes	HPLC, ELISA	3
Exhaled gases	GC	35
Lipid-DNA adducts	Fluorescence	36

measure of lipid peroxidation in simple *in vitro* systems, its application to complex biological fluids such as human plasma and subcellular systems has been limited. This is mainly because of its non-specificity and the potential confounding influence of various factors<sup>1,36-38</sup>.

### TBARS assay

TBARS assay can be performed by standard methods using MDA equivalents derived from tetraethoxypropane. MDA and other aldehydes have been identified as products of LP that react with TBA to give a pink coloured species that absorbs at 532 nm. The method involved heating of biological samples with TBA reagent for 20 min in a boiling water bath. TBA reagent contains 20% TCA, 0.5% TBA and 2.5 N HCl. After cooling, the solution was centrifuged at 2,000 rpm for 10 min and the precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm against a blank that contained all the reagents minus the biological sample. The MDA equivalents of the sample were calculated using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ . To reduce interference from iron released during homogenisation, it is advisable to include antioxidant/ iron-chelator like butylated hydroxytoluene (BHT) or EDTA<sup>28,29</sup>. The TBARS assay is simple and requires the reagent TBA, and dilute HCl. This was originally developed in 1958 for testing rancidity due to oxidized lipids in food material<sup>37</sup>. There are also several minor variations to the main components used for the TBARS assay.

### Interference in TBARS assay and remedies

Monitoring lipid peroxidation in complex conditions such as clinical or *in vivo* systems, calls for the use of methods that are sensitive, precise, and accurate, and are based on techniques that are robust and have proven inter-laboratory reproducibility. The TBARS assay was proposed over 40 years ago and is now the most commonly used method for measuring LP. However, as mentioned earlier, the assay is not specific for LP products. Besides aldehydic products that arise from LP, many other substances, including alkanals, protein, sucrose and urea may react with TBA to yield coloured species and thus contribute to overestimation of the extent of LP<sup>38</sup>. In plasma, it can react with sialic acid present in glycoproteins. In food materials, ketones, ketosteroids, acids, esters, sugars, proteins, pyridines, pyrimidines and vitamins can react with TBA and interfere with the assay. In the *in*

*vitro* experiments using subcellular fractions, blood components or tissue homogenates, sucrose, fructose, glucose, substituted pyrimidines, 2-deoxyribose, N-acetyl neuraminic acid, bilirubin, biliverdin, icteric serum and serum containing hemolysed erythrocytes can interfere with the colour development.

To correct for interference from sucrose, appropriate amounts can be added in the blank or test samples and the boiled samples with TBA can be extracted with butanol-pyridine solution<sup>39</sup>. Buffers made from Tris-maleate (up to 40 mM), imidazole (up to 20 mM), inorganic phosphate (up to 10 mM) and 4-morpholine propanesulfonic acid (up to 20 mM) did not affect the colour development. In plasma, the interference from sialic acid can be removed by carrying out the TBA reaction in phosphoric acid or TCA-HCl and correcting for absorption of sialic acid at 572 nm<sup>40</sup>.

There are potential artifacts in TBARS measurement in tissues from animals treated with antioxidants. There appears to be an initial (probably iron-induced) burst of peroxidation during homogenization, which is antioxidant susceptible. In addition, added TBARS products are formed during final heating stage in methods which retain membrane bound reactants throughout the assay, and this step is also susceptible to the presence of antioxidants<sup>41</sup>.

### Estimation of conjugated dienes

For estimating conjugated dienes test samples (tissue/membrane fractions) subjected to oxidative stress were treated with chloroform:methanol mixture (2:1) followed by vigorous vortexing and centrifugation at 2,000 rpm for 10 min. The upper layer obtained was discarded along with the proteins, while the lower chloroform layer was dried under a stream of nitrogen at 45°C. The residue obtained was dissolved in cyclohexane and absorbance was taken at 233 nm against a cyclohexane (standard 1 O.D. = 37.5 nmoles). The sensitivity of this assay is up to a few nanomoles (2 to 3 nmoles). This assay has to be carried out under inert atmosphere, in the presence of nitrogen or argon<sup>31</sup>.

### Estimation of lipid hydroperoxides

For LOOH estimation tissue samples exposed to damage by free radicals were treated with chloroform:methanol (2:1) mixture, followed by vigorous vortexing. The samples were centrifuged at 2,000 rpm for 10 min. The upper aqueous layer obtained was discarded along with the protein layer. The lower

chloroform layer was dried under a stream of nitrogen at 45°C. The residue obtained was treated with potassium iodide and incubated in the dark for 6-8 min. After incubation, acetic acid: chloroform mixture (3:2) was added followed by 0.25% cadmium acetate. After each step vortexing was done. The mixture was centrifuged at 2,000 rpm for 10 min. The upper layer was transferred into fresh tubes. Absorbance was read at 373 nm<sup>31</sup> (standard 1 O.D. = 86.89 nmoles). LOOH can also be estimated by a relatively simpler method using 'Fox reagent'<sup>42</sup>. This method can also be utilized for estimating oxidation of low-density lipoprotein (LDL). The FOX reagent consists of 250 µM ammonium sulphate, 100 µM xylenol orange, 25 mM H<sub>2</sub>SO<sub>4</sub> and 4 mM BHT in 90% (v/v) methanol. The working reagent should be routinely calibrated against solutions of H<sub>2</sub>O<sub>2</sub> of known concentrations. Samples under study (90 µl each) were mixed with 10 µl of methanol (in case of blank) or 10 µl of TPP (triphenyl phosphine) in methanol (in case of test samples). The samples were then incubated for 30 min at room temperature, followed by addition of 900 µl of FOX reagent. Once again the samples were incubated for 30 min, followed by centrifugation at 12,000 g for 10 min, prior to determination of absorbance at 560 nm. The level of lipid peroxide in the samples was determined by calculating the difference between the blank and test sample readings. Hydroperoxide content was determined by using a molar absorption co-efficient of  $4.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  in reference to H<sub>2</sub>O<sub>2</sub> standard curve. The sensitivity of LOOH assay is in the range of few nanomoles (2-5 nmoles). The assay has to be carried out under inert atmosphere and sometimes there will be interference, if there is excess iron present in the sample.

### Estimation of 4-hydroxynonenal

Considered a toxic second messenger, 4-hydroxy-2-nonenal (4-HNE) is one of the series of 4-hydroxy-2-alkenals formed during LP. These have diverse biological properties and varying ability to damage biological macromolecules, e.g., ~50-fold less concentrations of 4-HNE are required to produce a given level of protein adducts to those produced by MDA. Under conditions of neutral pH, 4-HNE is a significant an endogenous toxicant due to its greater electrophilicity.

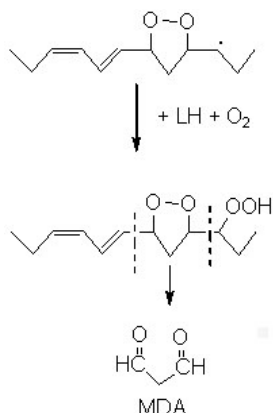
To measure this physiologically important aldehyde, test samples were suspended in 2.5 ml 5 mM phosphate buffer and centrifuged at 10,000 rpm

for 15 min. The pellet was incubated in 8 ml and supernatant in 2 ml 2.5 N dinitrophenylhydrazine (DNPH) (prepared in 1 N HCl) for 2 hr at 37°C. Then kept in ice for 1 hr (in dark). After incubation the lower layer was extracted in 2 ml dichloromethane by vortexing and centrifuged at 2,000 rpm for 10 min. The extracted layer was evaporated to dryness under nitrogen at 45°C. The residue thus obtained was dissolved in 0.5 ml dichloromethane. Of this sample, 0.15 ml was loaded onto a TLC plate. The TLC was carried out for the first 5 cm in dichloromethane and next 15 cm in benzene. The plates were dried in fume hood for 15 min. The bands were detected under U.V. light and marked at a resolution factor of 0.15. The marked bands were then scrapped off and transferred into fresh tubes followed by two extractions in 5 ml methanol with continuous vortexing and centrifuging at 2,000 rpm for 5 min. The methanol layer was evaporated to dryness under nitrogen at 45°C. The residue obtained was dissolved in 0.5 ml methanol and filtered using 0.5 µm filter, following which it was loaded onto HPLC. The wavelength of the detector was set at 478 nm. The column used was C18 bondapack, with methanol:water (0.8:0.2) and the flow rate were set at 1 ml/min<sup>43</sup>. Authentic 4-HNE from Sigma Chemicals was used as standard. The detection limit for the assay is 1 nmole. The assay is time-consuming and requires sophisticated and expensive instrumentation.

### Malondialdehyde

All unsaturated aldehydes may undergo further changes by autoxidation producing other volatile compounds. Thus, hydroperoxy aldehydes may undergo cleavage to give shorter chain aldehydes, sometimes with other chemical groups. Among these, MDA is of interest. Various precursors of MDA have been proposed, but the most probable and the most biochemically important seem to be the monocyclic peroxides formed from fatty acids with 3 or more double bonds. Other efficient sources have been described: hydroperoxy bis-epidioxides, hydroperoxy bis-cycloendoperoxides and dihydroperoxides. Diene fatty acids (linoleic acid) were also shown to be good precursors in some defined oxidative conditions (singlet oxygen, acid pH). As an example, 12-hydroperoxy linolenate may undergo a cyclization, followed by an oxidation forming a 5-membered hydroperoxy epidioxide as major product. The cleavage on each side of the endoperoxide ring was proposed as the main source of MDA. MDA may also

be formed in some tissues by enzymatic processes with prostaglandin precursors as substrates. Thus, thromboxane synthetase generates MDA, with thromboxane A<sub>2</sub>, from prostaglandin endoperoxides during human platelet activation<sup>44</sup>.



### Estimation of isoprostanes

Monitoring lipid peroxidation in clinical situations calls for the use of methods that are sensitive, precise and accurate, and are based on techniques, which are robust and have proven inter-laboratory reproducibility. In this context, the measurement of isoprostanes, which are specific peroxidation products of PUFAs, is now recognized as the most reliable index for the assessment of LP in humans and has been used to evaluate the clinical efficiency of antioxidant treatment in several diseases. Analysis of isoprostanes has been directed mostly towards measurement of the major F<sub>2</sub>-isoprostane, 8-epi-PGF<sub>2α</sub>, which is derived from arachidonic acid peroxidation. In humans, this can be measured in several biological fluids (plasma, urine, synovial fluid), using gas chromatography-mass spectrometry or commercially available enzyme immunoassay kits<sup>3</sup>. The detection limit for GC-MS estimation of isoprostanes is 50-100 pmoles per ml sample. The disadvantage of this assay is that it is expensive requiring expensive instrumentation. Estimation by Elisa kits is easier, but very expensive.

### Measurement of lipid-DNA adduct formation

Lipid-MDA adducts are important indicators of secondary damage formed due to by-products of LP. To measure these, DNA was isolated from whole hepatocytes or nuclei according to the method described by Marmur<sup>45</sup>. The isolated DNA was treated with proteinase K and RNase to remove the protein and RNA. The radioactivity associated with

DNA was determined by liquid scintillator. Fluorescence intensities at 410 and 460 nm excitation at 315 and 390 nm, respectively were used to quantify the amount of lipid-DNA adducts. Purified lipid-DNA adducts from experimental groups showed a fluorescence peak at 410 nm when excited with 315 nm<sup>36</sup>. Similar results have been obtained by Reiss in the reaction of calf thymus DNA with arachidonate<sup>46</sup>. Another product or products with excitation and emission maxima at 390 nm and 460 nm, respectively were also observed, but the yield was very low, similar to the observation in the reaction of DNA and MDA. The observed fluorescent products could be Schiff's base complexes formed by the reaction between amino groups of the DNA bases and the carbonyl end-products (including MDA and other carbonyls) of peroxidizing arachidonic acid and other polyunsaturated fatty acids. This assay though sensitive (detection limit 5 nmoles) is also quite expensive to perform.

### Measurement of exhaled gases

Breath alkanes were measured as an index of oxidative stress in human subjects<sup>47</sup>. Subjects breathed for 4.0 min through a cardboard mouthpiece connected to a Rudolph valve from a Tedlar bag (Aerovironment, Monrovia, CA, USA) containing hydrocarbon-free air (HCFA) (0.1 ppm total hydrocarbons expressed as methane, Ultra-Zero Air, Prax Air, Canada). The exhaled air was discarded. Thus, the initial atmospheric air with its hydrocarbon contents was flushed from the lungs. During the succeeding 2.0 min, while the HCFA was inspired, breath was collected into a second Tedlar bag for alkane measurements. The volume of air collected was recorded by a Medishield spirometer attached to the output portion of the Rudolph valve. Throughout the 6 min period, the nose was closed by a clip.

The analysis of breath for alkanes was performed according to the method described by Lemoyne *et al.*<sup>48</sup> on a Shimadzu GC 14A gas-solid chromatograph (Mandel Scientific Company Limited, Guelph, Ontario) set at range 4 and sensitivity 104 and equipped with a flame ionization detector (FID). Hydrocarbon-free nitrogen gas was used as a carrier at a flow of 20 ml/min. Pentane and ethane gases were analyzed in a column filled with Porasil-D (Chromatographic Specialties Inc, Brockville, ON, Canada) in a stainless steel coil held isothermally at 60°C with a calibration curve derived from known concentrations of pentane and ethane standard (1 ppm

ethane; 1 ppm pentane, Prax Air). One flame of double FID was used, at 200°C, with hydrogen at a flow rate of 30 ml/min (0.5 kg/cm<sup>2</sup>) and medical compressed air at a flow rate of 0.8 L/min for maximum sensitivity. Finally, the output of the FID was led to a recorder (Shimadzu CR 501 Chromatopac, Mandel Scientific Company, Ltd.). The peak areas under the curve for ethane and pentane were measured using 50 ml of the breath sample. The concentrations of ethane and pentane production were then calculated and expressed in pmol/kg/min. The assay of volatile hydrocarbons is mainly applicable to *in vivo* conditions on large amount of samples. The assay is also cumbersome and requires specialist equipment and is also subject to artifacts<sup>49</sup>.

### Estimation of other products of lipid peroxidation

Lipid-protein adducts, lipofuscin pigments etc. can also be monitored by specific methods<sup>50</sup>. Free fatty acids and their oxidation products can be measured by HPLC<sup>51</sup>. Exhaled gases such as pentane, ethane etc. can be measured by gas chromatography. This can be used as an '*in vivo*' indicator of lipid peroxidation in man and laboratory animals. The technique is now available for study conditions, such as cystic fibrosis, in which oxidative stress component in tissue injury is being implicated<sup>52</sup>.

### Other methods for lipid peroxidation products

The following parameters also can be used as a measure of lipid peroxidation: oxygen uptake, as assayed by oxygraph or oxystat. Membrane alteration can be measured by electrophoresis using various markers or supports<sup>53</sup>. Chemiluminescence of materials generated during LP, such as triplet carbonyls and other excited states can be monitored by special equipments that monitor such products at specific wavelengths<sup>54</sup>. Fluorescent products that are formed by adduct formation can also be assessed. An indirect way of measuring LP is by monitoring the consumption of antioxidants, such as vitamin E<sup>1,53</sup>.

To measure products of LP, a large number of approaches can be used. These include spectrophotometry, spectrofluorimetry, HPLC, GC-MS combined with selective ion monitoring, gas chromatography, chemiluminescence, ELISA, electrospray combined with mass spectrometry, oxygraph-oxystat, photoionization gas chromatography, UV-IR spectroscopy, GC-ECM-MS, single photon counting, fast atom bombardment and tandem MS, fourier transform infrared spectroscopy and electrophoresis.

Among the modern techniques, the one that is gaining popularity is electrospray-MS, a soft ionization technique, which does not result in fragmentation of biomolecules, thus allowing the study of phospholipids keeping the structures intact. This method also is used for detection of isoprostanes and oxidized free fatty acids<sup>55</sup>. GC-MS is a very sensitive method for the analysis of aldehydes, fatty acids and their hydroperoxides, lipid hydroperoxides and isoprostanes<sup>3</sup>.

### Concluding remarks

Lipid peroxidation is a fascinating field. It forms the basis of reactions of essential fatty acids. There is much more to be done than TBARS reaction and its inhibition. Many questions and pervading myths remain to be tackled. The story of lipid peroxidation along with PUFA will have many surprises and benefits in a number of conditions and modern diseases that unfolded with adapting to franchised food chain.

### Acknowledgement

The first two authors wish to thank Dr K P Mishra, Head, Radiation Biology & Health Sciences Division, Bhabha Atomic Research Centre, Mumbai for his encouragement, Miss Jai C Tilak for helping in the literature survey and Mrs. Jayashree K Bloor for going through the manuscript.

### References

- 1 Rice-Evans C & Burdon R (1993) *Prog Lipid Res* 32, 71-110
- 2 Raha S & Robinson B H (2000) *Trends Biochem Sci* 25, 502-508
- 3 Yoshikawa T, Toyokuni S, Yamamoto Y & Naito Y (eds.) (2000) *Free Radicals in Chemistry Biology and Medicine*, OICE International, Saint Lucia, London
- 4 Wills E D (1969) *Biochem J* 113, 315-325
- 5 Sies H (1996) (ed.) *Antioxidants in Disease, Mechanisms and Therapy*, Academic Press, New York
- 6 Pfeifer P M & McCay P B (1972) *J Biol Chem* 247, 6763-6769
- 7 Halliwell B & Gutteridge J M C (1997) in: *Free Radicals in Biology and Medicine*, Oxford University Press
- 8 Wallace D C (1999) In: *Frontiers Cell Bioenergetics*, (Papa S, Guerrieri F & Tager J M, eds.), Kluwer Academic/Plenum Publishers, New York
- 9 Yagi K (1987) *Chem Phys Lipids* 45, 337-351
- 10 Castranova V & Vallyathan V (2000) *Environ Health Perspect* 108, 4675-4684
- 11 Cuzzocrea S, Riley D P, Caputi A P & Salvemini D (2001) *Pharmacol Rev* 53, 135-159
- 12 Parola M, Bellomo G, Robino G, Barrera G & Dianzani M U (1999) *Antiox Redox Signaling* 1, 255-283
- 13 Esterbauer H, Schaur R J & Zollner H (1991) *Free Radic Biol Med* 11, 81-128



- 14 Park J W & Floyd R A (1992) *Free Radic Biol Med* 12, 245-250
- 15 Akasaka S (1984) *FEBS Lett* 172, 231-234
- 16 Porter N A, Wolf R A, Yarbrow E M & Weenen H (1979) *Biochem Biophys Res Commun* 89, 1058-1064
- 17 Porter N A, Bruce A, Weber H W & Jamil A K (1980) *J Am Chem Soc*, 102, 5597-5601
- 18 Packer L & Ong A S H (eds.) (1998) *Biological Oxidants and Antioxidants: Molecular Mechanisms and Health Effects*, AOCS Press, Champaign, Illinois
- 19 Ramasarma, T (2000) *Toxicol* 148, 85-91
- 20 Wills E D (1971) *Biochem J* 123, 983-991
- 21 Schich K M (1980) *CRC Critical Rev Food Sci Nutr* 13, 189
- 22 Fishwick M J & Swoboda P A T (1977) *J Sci Food Agric* 28, 387-391
- 23 Yamamoto Y, Haga S, Niki E & Kamiya Y (1984) *Bull Chem Soc Jpn* 52, 1260-1264
- 24 Barclay L R C, Locke S J, MacNeil J M, VanKessel J, Buton G W & Ingold K U (1984) *J Am Chem Soc* 106, 2479-2481
- 25 Rojas Wahl R U, Zeng L, Stephen S A, Madison R L, DePinto B J & Shay B J B (1998) *J Chem Soc Perkin Trans 2*, 2009-2017
- 26 Stocker P, Lesgards J, Vidal N, Chalier F & Prost M (2003) *Biochim Biophys Acta* 1621, 1-8
- 27 Fessel J P, Porter N A, Moore K P, Sheller J R & Roberts L J (2002) 2nd. *Proc Natl Acad Sci USA* 99, 16713-16718
- 28 Nelson S K, Bose S K & McCord (1994) *Free Radic Biol Med* 16, 195-200
- 29 Devasagayam T P A, Pushpendran C K & Eapen J (1983) *Biochim Biophys Acta* 750, 91-97
- 30 Hunter F E, Gebicki J M, Hoffsten P E, Weinstein J & Scott A (1963) *J Biol Chem* 238, 828-835
- 31 Buege J A & Aust S D (1978) *Methods Enzymol* 52, 302-310
- 32 Kamat J P & Devasagayam T P A (1996) *Chem-Biol Interact* 99, 1-16
- 33 Kamat J P & Devasagayam T P A (1995) *Neurosci Lett* 195, 179-182
- 34 Esterbauer H, Eckl P & Orterer A (1990) *Mutat Res* 238, 223-233
- 35 Burk R F & Ludden T M (1989) *Biochem Pharmacol* 38, 1029-1032
- 36 Cao E H, Liu X Q, Wang L G, Wang J J & Xu N F (1995) *Biochim Biophys Acta* 1259, 187-191
- 37 Sinnhuber R O, Yu I C & Yu Te C (1958) *Food Res* 23, 620.
- 38 Jardine D, Antolovich M, Prenzler P D & Robards K (2002) *J Agric Food Chem* 50, 1720-1724
- 39 Shlafer M & Shepard B M (1984) *Anal Biochem* 137, 269-276
- 40 Lapenna D, Ciofani G, Pierdomenico S D, Giamberardino M A & Cuccurullo F (2001) *Free Radic Biol Med* 31, 331-335
- 41 Lin Y & Jamieson D (1994) *Biomed Environ Sci* 7, 79-84
- 42 Nourooj-Jadeh J, Tajaddini-Sarmadi J, Eddie Ling K L & Wolff S P (1996) *Biochem J* 313, 781-786
- 43 Esterbauer H & Cheeseman K H (1990) *Methods Enzymol* 186, 407-421
- 44 Hecker M & Ullrich V (1989) *J Biol Chem* 264, 141-150
- 45 Marmur J (1961) *J Mol Biol* 3, 205-218
- 46 Reiss U & Tappel A L (1973) *Biochim Biophys Acta* 312, 608-615
- 47 Aghdassi E A & Allard J P (2000) *Free Radic Biol Med* 28, 880-886
- 48 Lemoyne M, Van Gossum A, Kurian R, Ostro M, Axler J & Jeejeebhoy K N (1987) *Am J Clin Nutr* 46, 267-272
- 49 PUNCHARD N A & KELLY F J (eds.) (1996) *Free Radicals: A Practical Approach*, Oxford Univ. Press, New York
- 50 Dillard C J & Tappel A L (1984) *Methods Enzymol* 105, 337-341
- 51 Frankel E N (1999) *Methods Enzymol* 299, 190-201
- 52 Lawrence G D & Cohen G (1984) *Methods Enzymol* 105, 305-311
- 53 Van Ginkel G & Sevanian A (1994) *Methods Enzymol* 233, 273-288
- 54 Murphy M E & Sies H (1990) *Methods Enzymol* 186, 595-610
- 55 Morrow J D & Roberts L J (1999) *Methods Enzymol* 300, 3-12