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ASPECTS TO CONSIDER WHEN DETECTING AND MEASURING LIPID PEROXIDATION†

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1. INTRODUCTION

"Initiation" is a term used to describe the abstraction of a hydrogen atom from a polyunsaturated fatty acid (PUFA) which leads to the propagating chain reactions characteristic of lipid autoxidation. Primary products of lipid autoxidation (peroxidation) are peroxides, and the secondary products derived from the peroxides are carbonyl compounds (Figure 1).

Any free radical with sufficient reactivity to abstract a hydrogen atom from a PUFA has the potential to initiate lipid peroxidation. The hydroxyl radical ($OH^{\cdot}$) can initiate lipid peroxidation whereas superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) cannot. However, when $O_2^{\cdot -}$ and H_2O_2 are added to lipids they appear to stimulate lipid peroxidation. Ferrous ions autoxidise in aqueous solution to form $OH^{\cdot}$ radicals and when added to lipids readily stimulate lipid peroxidation although this peroxidation is not inhibited by scavengers of the $OH^{\cdot}$ radical[1]. How then can these findings be explained?

(A) All laboratory reagents and preparations contain micromolar concentrations of iron salts sufficient to catalyse radical reactions[2].

(B) All biological and commercial lipid preparations contain from trace to large amounts of lipid peroxides[3].

Both $O_2^{\cdot -}$ and H_2O_2 can reduce and recycle traces of iron salts to the reactive ferrous state. It is the ferrous ion which is highly stimulatory towards lipid peroxidation.

Further, ferrous ions *in vitro* appear to react more readily with lipid hydroperoxides (equation 1) than with molecular oxygen (Equation 2)[4].

$$2LOOH \xrightarrow{Fe\ salts} LO^{\cdot} + LO_2^{\cdot} + H_2O \qquad \text{(equation 1)}$$

$$Fe^{2+} + O_2 \rightleftharpoons Fe^{2+}\text{-}O_2 \rightleftharpoons Fe^{3+} + O_2^{\cdot -} \qquad \text{(equation 2)}$$

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

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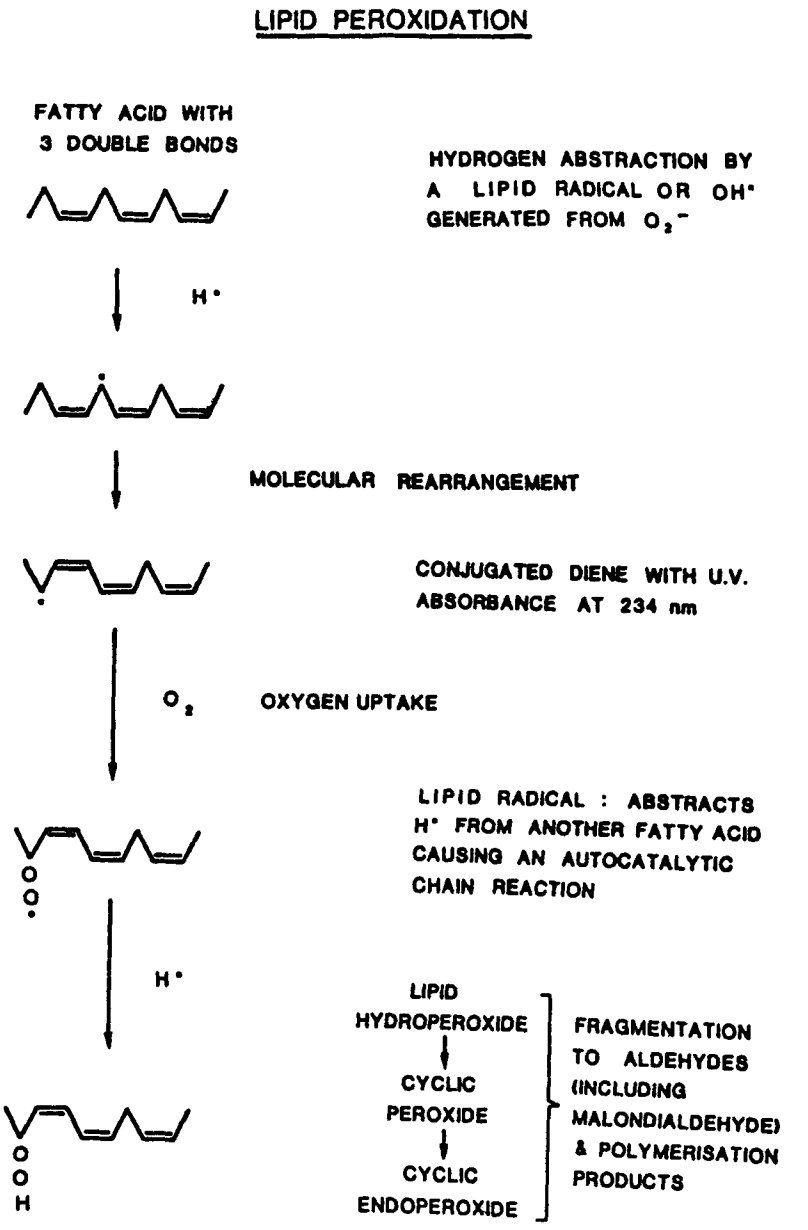


FIGURE 1

Alkoxyl ($LO^{\cdot}$) and peroxyl ($LO_2^{\cdot}$) radicals are able to abstract $H^{\cdot}$ from PUFAs and continue lipid peroxidation.

2. EARLY STAGES OF LIPID PEROXIDATION

Diene Conjugation

Following abstraction of a hydrogen atom from a PUFA a bond rearrangement takes place giving rise to a carbon-centred radical with a conjugated diene structure (Figure 1). An increased UV absorption is seen at around 230–235 nm (Figure 2).

Following oxygen uptake (Figure 1) which can be measured using a Clark-type electrode, there is a further and rapid increase in diene conjugation as autoxidation of lipid occurs. Diene conjugation is a sensitive measure of lipid peroxidation in bulk lipids but, because many biological molecules have a tendency to absorb in the UV, it must be combined with separative techniques for meaningful biological application.

3. INTERMEDIATE STAGES OF LIPID PEROXIDATION

The thiobarbituric acid test

The thiobarbituric acid (TBA) reaction was introduced into biology by Kohn and Liversedge in 1944[5]. Sinnhuber and colleagues (1958) later suggested that the characteristic colour reaction given by oxidised lipids and TBA (A532 nm) was a product of the reaction of 2 molecules of TBA with one molecule of malondialdehyde (MDA) (Figure 3)[6]. This has recently been confirmed using techniques of high-field NMR[7].

Widespread use of the TBA reaction by the food and dairy industries established the TBA test as an important test of lipid rancidity. However, most users of the test found little, if any, "free" MDA in their test systems and it was therefore assumed that most of the MDA was derived from the breakdown of peroxides during the acid-heating stage of the test — an important point that seems to have been forgotten by many contemporary users of the test.

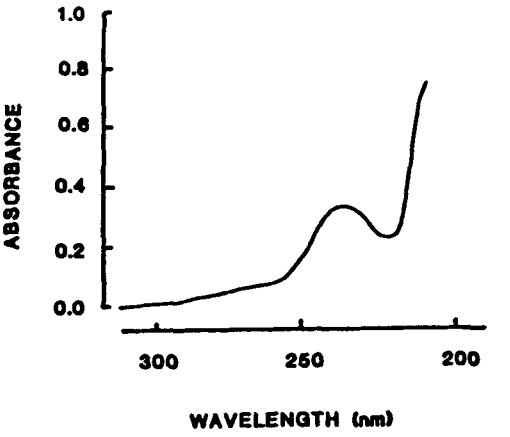


FIGURE 2 Diene conjugation of oxidised PUFA.

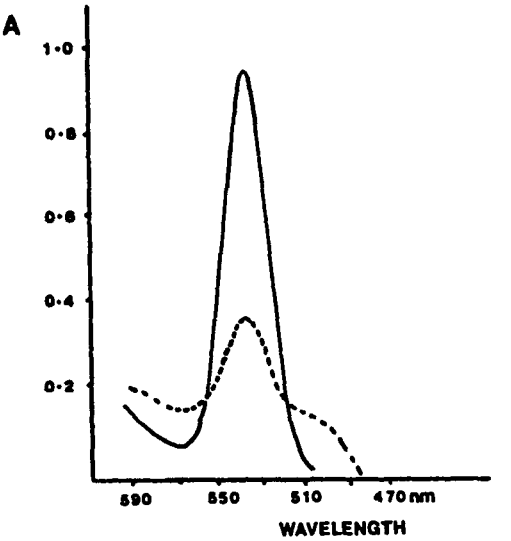


FIGURE 3 TBA-reactivity --- oxidised PUFA, —— MDA standard.

3.1. *How many different TBA tests are there?*

The TBA test consists of merely heating bulk lipids or biological material with acid and TBA. The simplicity of the test has led to many different versions of the test being adopted. A comparison of results between laboratories is therefore extremely difficult.

3.2. *What factors affect the TBA test?*

When using the TBA test it must be remembered that the biological sample under examination will be subjected to a severe chemical reaction before results are obtained. Heating at 100°C in acid brings about substantial changes in the sample and its lipids. Since little "free" MDA is usually present in biological samples, an essential requirement of the TBA test is to maximally release MDA from precursor molecules ie, lipid peroxides. The autoxidation mechanism proposed for the formation of MDA from PUFAs by Dahle, Hill and Holman[8] was recently revised by Pryor and colleagues to include the bicyclic endoperoxides[9]. The decomposition of peroxides to MDA, via peroxy radicals, requires traces of iron salts[10], and iron has long been known to influence the performance of the TBA reaction[11,12]. Peroxy radicals formed during the TBA reaction can abstract H· atoms from PUFAs to start a chain reaction giving rise to more TBA reactive material[10,13]. To prevent peroxidation during the TBA reaction therefore, antioxidants have been added to the TBA reagents[13] by some workers.

Preparation of the sample, by extracting lipids or precipitating proteins, can substantially alter the performance of the TBA test. However, in our laboratory we prefer to heat the whole untreated biological sample without the addition of antioxidants, chelators or iron salts, and to use the test as a measure of the potential of the material to undergo the chain reactions of lipid peroxidation.

CAREFULLY CONSIDER IN YOUR SYSTEM:—

3.2.1. The effect of iron salts. They can stimulate your incubation reaction as well as your TBA test.

3.2.2. The effect of antioxidants and metal chelators. They can inhibit your incubation reaction as well as your TBA test.

3.2.3. The preparation of your material. Peroxidation can occur during the acid-heating stage of the TBA test.

3.2.4. The acid you use for the TBA test. Different MDA precursors require different acid conditions for their breakdown to MDA[14].

3.2.5. Rigorous controls. Acetic acid used in the TBA test can contain high amounts of TBA-reactive material[15].

3.3. How sensitive and specific is the TBA test?

When the TBA test is applied to bulk lipids and biological samples its sensitivity is high because the test amplifies oxidative changes that have begun in the sample. If it only measured "free" MDA it would be of little, or no, use as a sensitive measure of lipid peroxidation.

Since many different peroxidic intermediates release MDA[16] it is not specific for any stage of lipid peroxidation. Further, many non-lipid molecules directly react with TBA to form chromogens[15,17]. Others such as aminoacids, carbohydrates, nucleic acids and benzoate are TBA-reactive after damage by free radicals[14,18]. Bile pigments, which often occur in plasma, are TBA reactive and their absorption spectra is shown in Figure 4.

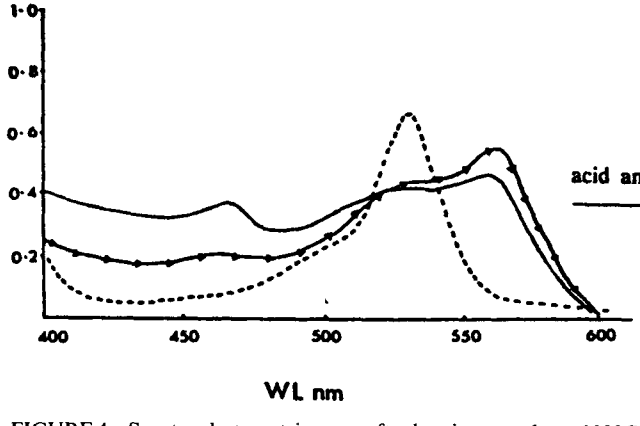
Interference from many biological substances can be avoided by careful sample preparation[19] or by measuring the fluorescent properties of the MDA-TBA complex[20,15]. Figure 5 shows the fluorescent properties of the MDA-TBA complex.

Only dilute solutions of the MDA-TBA complex can be measured by fluorescence, since the complex shows substantial inner-filter effects. Fluorescence will appear to fall as the concentration of complex increases. Concentration ranges must first be established to ensure linearity. Fluorescence is expressed in relative fluorescence intensity units (RFI) against a standard substance with a known fluorescence similar to the test material. Rhodamine B has been used by the author's laboratory for this purpose.

3.5. The effect of pH on the MDA-TBA complex and on MDA

The MDA-TBA complex can only be formed at acid pH. However, once formed its absorption spectra can be changed from 532 to 545–555 nm by adding alkali (Figure 6). The acid TBA chromogen is readily extractable into butan-1-ol, a procedure useful for eliminating turbidity.

MDA for use as a standard is usually prepared by the acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP) or 1,1,3,3,-tetraethoxypropane (TEP)[21]. However, under the conditions described by Kwon and Watts[21] hydrolysis may not be com-



Spectrophotometric scans after heating samples at 100°C for 15 min with acetic acid and TBA reagent. ---------- MDA standard, ▶—▶—▶—▶—▶— jaundiced serum, ——— 10μM biliverdin standard.

FIGURE 4 Spectrophotometric scans after heating samples at 100°C for 15 min with acetic acid and TBA reagent. -------- MDA standard, ▶—▶—▶—▶—▶— jaundiced serum, ——— 10 μM biliverdin standard.

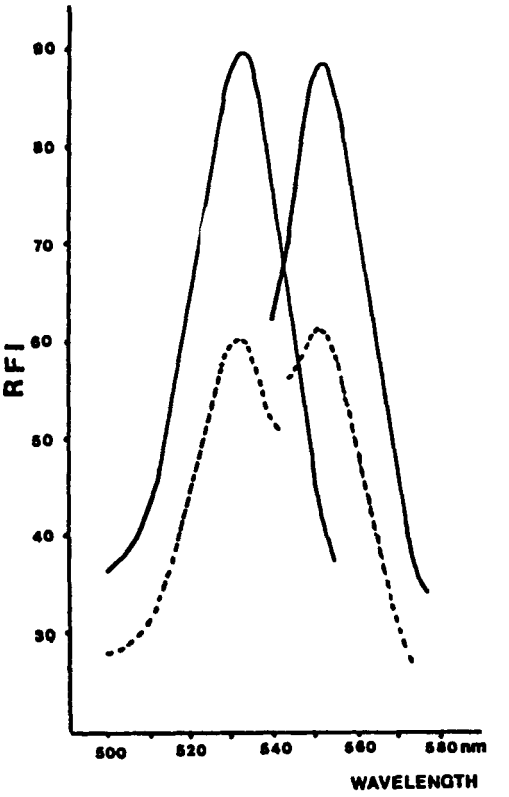
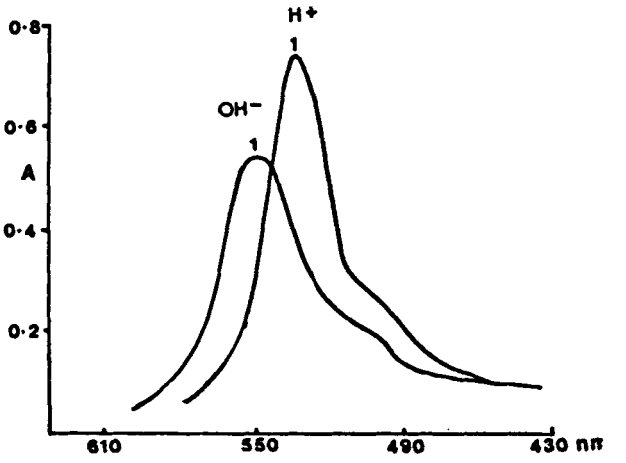


FIGURE 5 Fluorescent properties of the MDA-TBA complex.



pH-dependant visible spectrum of TBA adduct.

FIGURE 6 pH-dependant visible spectrum of the MDA-TBA complex.

plete and substantial polymerization of MDA (polyMDA) can occur[22]. An alternative hydrolysis procedure is to shake TMP with Dowex 50 resin (H^+ form)[23]. "Free" MDA if present will react with TBA at room temperature under any conditions of acidity[14].

MDA shows a distinctive UV absorption spectra (Figure 7). At pH values of 7.0, or greater, it absorbs at 267 nm (enolate form), whereas below pH 3.0 it absorbs at 245 nm (cyclic form).

4. LATE PRODUCTS OF LIPID PEROXIDATION

The breakdown of peroxides will yield a vast number of secondary carbonyl compounds including MDA[24]. These are of interest as products of lipid peroxidation which can be measured, as well as molecules with profound biological activities[25,26]. Tappel and his colleagues showed that dialdehydes such as MDA react with primary amino groups of proteins, peptides, aminoacids, DNA and phospholipids to form highly fluorescent Schiff bases (aminoiminopropenes)[27,28]. These have fluorescent properties similar to the so-called "age pigments" studied by microscopists for nearly 150 years. When the amino donor is water soluble, a polar fluorescent complex is formed. When a phospholipid is involved, a non-polar fluorescent complex is formed. Figure 8 shows the formation of non-polar fluorescence during the peroxidation of bovine brain phospholipids.

It has been observed that fluorescence can form in peroxidised fatty acids when no amino groups are present[29,30]. One product contributing to this fluorescence has been shown to be polymerised MDA[31]. Figure 9 shows the fluorescent spectra of polyMDA.

It is possible to make fluorescent Schiff bases from peroxidised fatty acids by replacing the TBA reagent with serine[31]. See Figure 8.

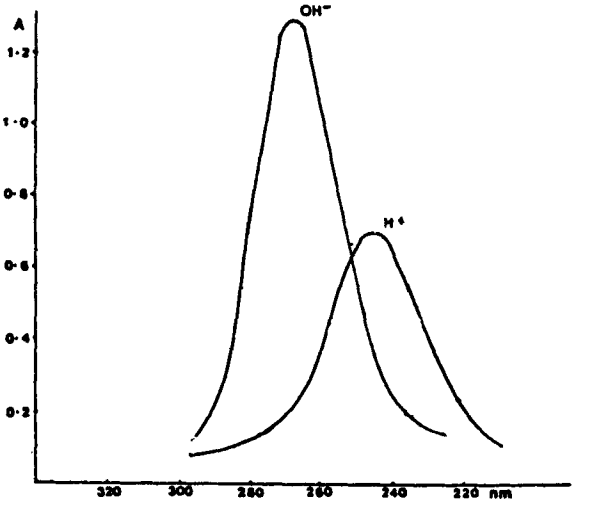


FIGURE 7 pH-dependant ultraviolet absorption spectrum of MDA.

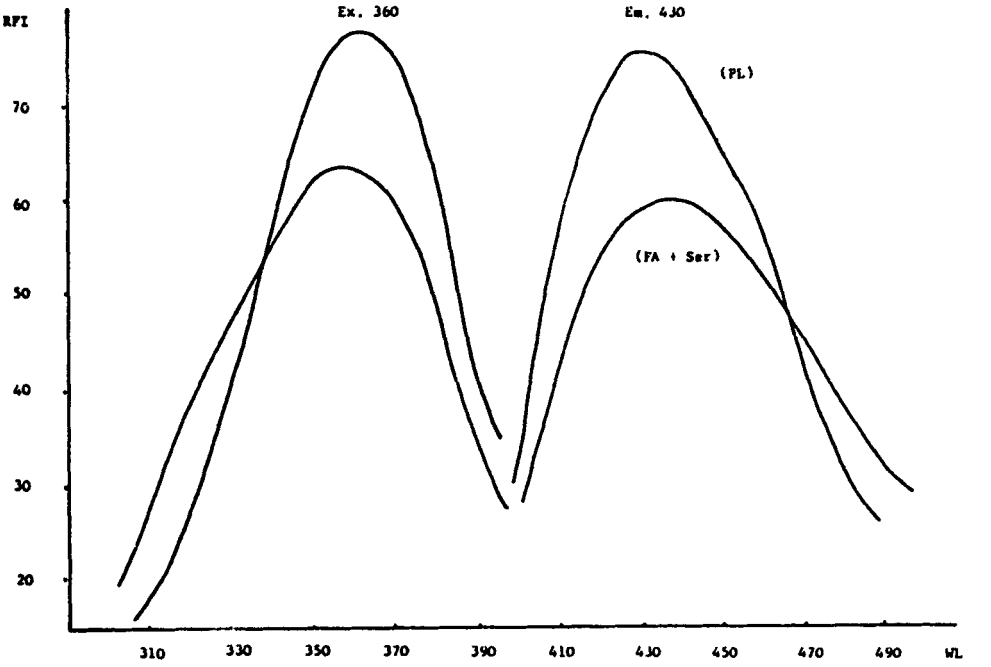


FIGURE 8 Fluorescent scans at excitation 360 nm emission, 430 nm of (a) autoxidised phospholipid (PL), and (b) autoxidised fatty acid (FA) heated with serine (Ser).

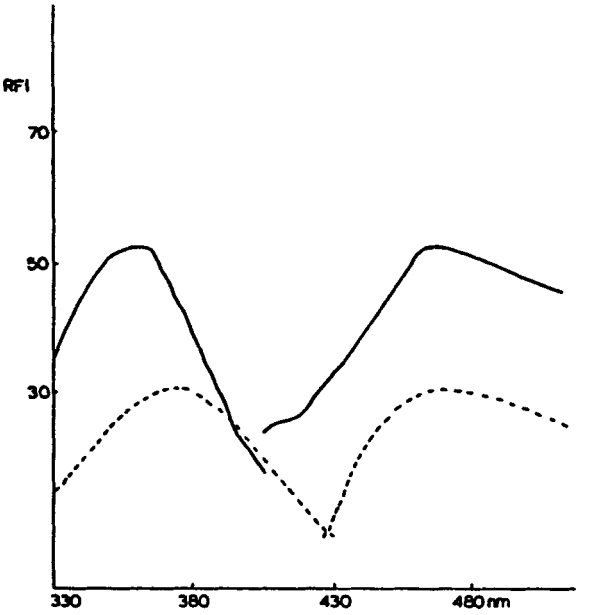


FIGURE 9 Fluorescence scans of polymerized malondialdehyde prepared from 1,1,3,3-tetramethoxypropane and arachidonic acid lipid peroxides decomposed by heating. RFI, relative fluorescence intensity. ———, Polymalondialdehyde. Excitation, 360 nm; emission, 470 nm; filter, 430 nm; slits, 10 nm; sensitivity, ×0.3. ------, Decomposed peroxide from autoxidized arachidonic acid. Excitation, 375 nm; emission, 470; filter, 430 nm; slits, 10 nm; sensitivity, ×0.3.

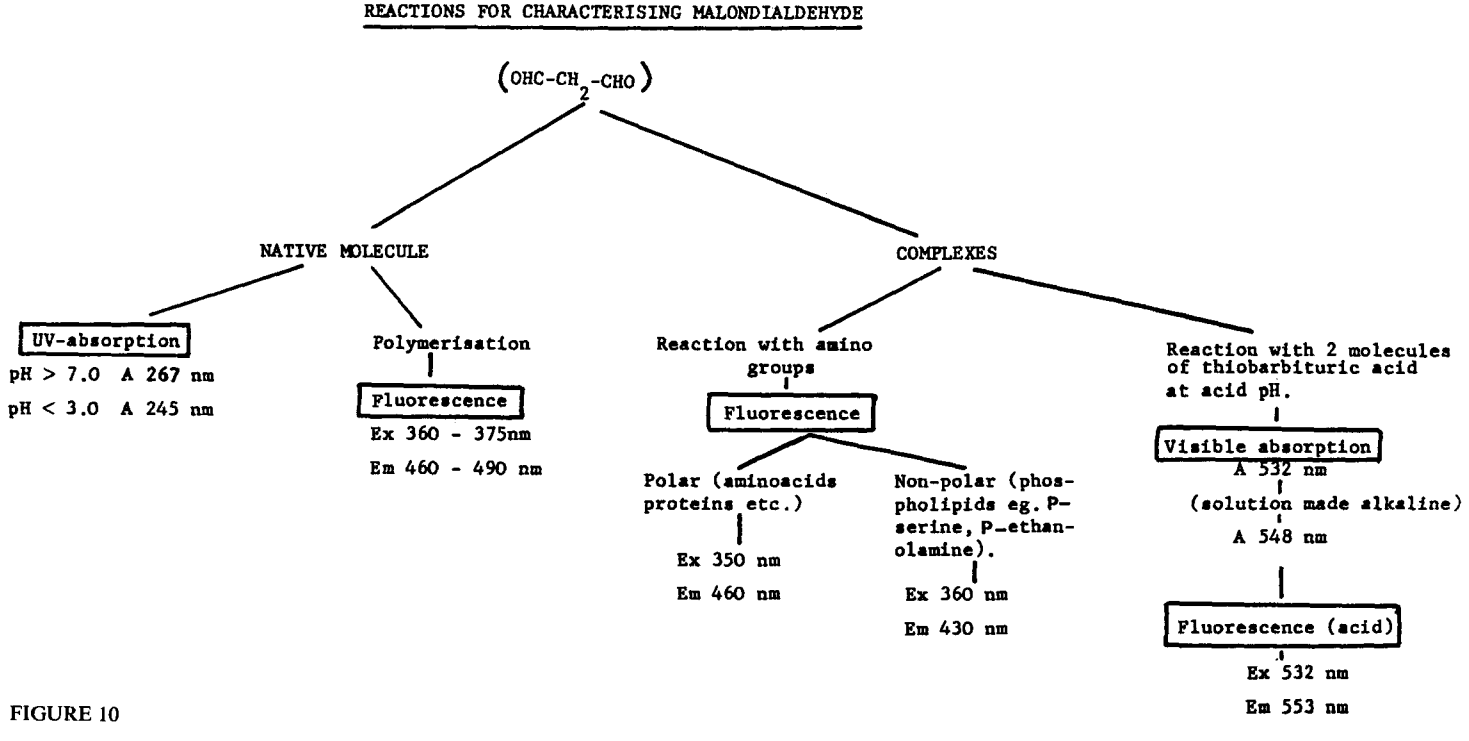


FIGURE 10

The direct measurement of Schiff base fluorescence is extremely sensitive although it represents only a small fraction of total peroxidation products. One great advantage of its use is, however, that it measures only the changes which have occurred *in vivo* or during the incubation process. It also indicates that MDA is formed during peroxidation both *in vivo* and *in vitro* and that this rapidly reacts with any amino groups present.

Figure 10 summarises the reactions for characterising malondialdehyde formed by decomposing lipid peroxides or "free" in solution.

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