Biochemical Pharmacology 1961, Vol. 7, pp. 7–16. Pergamon Press Ltd., Printed in Great Britain.

EFFECT OF UNSATURATED FATTY ACIDS AND THEIR PEROXIDES ON ENZYMES

E. D. WILLS

Department of Biochemistry, Medical College of St. Bartholomew's Hospital, London, England.

(*Received* 19 *August* 1960)

Abstract—Many enzymes of various types, e.g. urease, glyoxalase and papain, have been found to be inhibited by emulsions of oxidised unsaturated fatty acids such as linoleic or linolenic. Some enzymes, such as catalase and D-amino acid oxidase, were unaffected. In most cases, the extent of the inhibition was dependent on the peroxide value of the emulsion, and on the time of contact of enzyme and emulsion. In general, —SH enzymes were found to be more readily inhibited than enzymes not possessing —SH groups and to be protected by sulphydryl compounds, such as glutathione.

INTRODUCTION

CERTAIN unsaturated fatty acids such as linoleic, linolenic and arachidonic have been known for many years to be dietary essentials for mammals. The precise function of these acids is not yet clear, although they are known to be combined as phospholipids and form part of essential cell components such as the cell membranes.

During the past few years, interest in these fatty acids has been stimulated for two main reasons; firstly on account of the relationship of unsaturated fatty acids to cholesterol metabolism,[1] and secondly on account of the fact that peroxides formed from unsaturated fatty acids may be responsible for radiation damage.[2]

Several investigators have shown that there is a connexion between peroxide formation and radiation damage to mammals. Thus Horgan *et al.*[3] found that peroxides formed from linoleic acid were toxic when injected into mice. Initially, these toxic effects were considered to be similar to the effects of radiation, but toxic effects resulting from the administration of fatty acid peroxides have subsequently been shown to develop much more rapidly.[4] However, Muset, Esteve and Mateu,[5] found that lipoxidase, which oxidises unsaturated fatty acids to peroxides, was toxic when injected into or fed to mice. Many of the lipoxidase effects were found to be very similar to radiation effects, and these workers classed lipoxidase as radiomimetic. Other organic peroxides have also been shown to be radiomimetic.[6]

The toxicity of fatty acid peroxides must be the result of some biochemical damage, but as yet few studies have been made of the effect of these peroxides on vital cellular processes. Bernheim, Wilbur and Kenaston[7] have shown, however, that fatty acid peroxides inhibit some oxidative enzymes and Dubouloz and Fondarai[8] found that ethyl oleate peroxide was capable of oxidising the —SH groups of proteins to —S—S— and to further oxidation products. More recently, Tappel and Zalkin[9] found that liver mitochondria took up oxygen in the absence of added substrate and

that this oxygen uptake resulted in the formation of lipid peroxides. During the formation of these peroxides there was a concomitant destruction of some of the mitochondrial enzymes, e.g. succinic oxidase and DPNH-cytochrome c reductase.

The effect of emulsions of oxidised unsaturated fatty acids has now been examined on a wide variety of enzymes. It has been found that peroxide inhibition is not confined to oxidative enzymes and that several enzymes of other classes, e.g. hydrolytic enzymes, are inhibited. Furthermore, certain oxidative enzymes have been found to be resistant to peroxide inhibition. In general, sulphydryl enzymes have been found to be much more strongly inhibited by peroxides than enzymes not possessing —SH groups.

A preliminary account of some of this work has already been published.[10]

MATERIALS AND METHODS

Fatty Acids. Oleic acid, palmitic acid and stearic acid were pure samples obtained from B.D.H. Ltd.. Pure linoleic acid was obtained from the California Research Foundation. Pure linolenic acid was obtained from Mann Research Laboratories, New York, U.S.A..

Mixed linseed acids. These were prepared by the hydrolysis of linseed oil using the method described by Hilditch.[11] An analysis of these acids on a Gas Chromatogram showed that the mixture contained approximately 45% linolenic acid, 20% linoleic acid, 12% oleic acid and 23% saturated acids. The iodine number was found to be 188. A value of 280 was taken for the average molecular weight.

Fatty acid emulsions. For the majority of experiments the fatty acid was dissolved in ethanol to give an 0·1 M solution. 1 ml of this solution was then added to 9 ml 0·05 M phosphate buffer (pH 7·4) and the mixture was quickly shaken to produce a stable emulsion containing 10% ethanol and 0·01 M fatty acids. When weaker concentrations of fatty acid were used, a clear solution was obtained. Control experiments using buffer containing 10% ethanol were always carried out when testing the effect of these emulsions on enzymes. For some experiments, emulsions were prepared by dissolving the required quantity of fatty acid in an equivalent amount of ammonium hydroxide or sodium hydroxide.

Enzyme preparations and activity measurements. The methods of preparation and determination of the enzymes used have been previously described by Wills.[12, 13, 14, 15]

RESULTS

Comparison of the effects of saturated and unsaturated fatty acids on enzymes

The effect of emulsions containing 2×10^{-3} M palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, or mixed linseed acids in 0·05 M pH 7·4 phosphate buffer was examined on 18 different enzymes. In each case the enzyme was incubated with the emulsion for 20 min at 37° before determining the activity.

Under these conditions the enzymes examined were found to be divisible into four groups:

A. Those unaffected by either saturated or unsaturated fatty acids:

B. Those inhibited by saturated and unsaturated fatty acids to an approximately equal extent;

C. Those unaffected by saturated fatty acids but inhibited by unsaturated fatty acids;

D. Those inhibited to a greater extent by unsaturated than by saturated fatty acids.

The enzymes tested are shown listed in their various groups in Table 1. In Table 2 specific figures are given for the inhibition of representative members of each group by equivalent concentrations of palmitic and linoleic acid emulsions. Stearic acid emulsions were always found to behave exactly as those of palmitic acid and emulsions of oleic acid resembled those of linoleic acid but their inhibitory action was always very much weaker.

TABLE 1. COMPARISON OF THE EFFECTS OF PALMITIC ACID EMULSION (2×10^{-3} M) AND LINOLEIC ACID EMULSION (2×10^{-3} M) ON SOME ENZYMES

Group	Enzymes		Palmitic acid emulsion	Linoleic acid emulsion	Comment
A	Xanthine oxidase Tyrosinase	Catalase D-Amino acid oxidase	No effect	No effect	
B	Cholinesterase Serum esterase Lipase	Invertase Pepsin	Inhibited	Inhibited	Inhibition by two acids approximately equal
C	Glyoxalase (—SH) Choline oxidase (—SH)	β-amylase (—SH) Liver respiration (—SH)	No effect	Inhibited	
D	Succinic oxidase (—SH) Cytochrome oxidase	Trypsin Papain (—SH) Urease (—SH)	Inhibited	Inhibited	Inhibition by linoleic acid much greater than by palmitic acid

TABLE 2. A COMPARISON OF THE EFFECTS OF PALMITIC ACID EMULSION (2×10^{-3} M) AND LINOLEIC ACID EMULSION (2×10^{-3} M) ON REPRESENTATIVE ENZYMES OF GROUPS A, B, C AND D

Group	Enzyme	Inhibition, per cent	
		Palmitic acid (2×10^{-3} M)	Linoleic acid (2×10^{-3} M)
A	Catalase	0	0
B	Serum esterase	32	32
C	Glyoxalase	0	83
D	Papain	21	86

Group classifications are described in Table 1 and the text.

The pattern of the inhibition of enzymes of Group D was changed when dilute, e.g. 4×10^{-4} M, emulsions of palmitic acid and linoleic acid were compared. At this concentration palmitic acid had virtually no effect, whilst linoleic acid was strongly inhibitory (Fig. 1).

The effect of emulsions of unsaturated fatty acids, such as linoleic or linolenic, on the enzymes of groups C and D was found to be mainly dependent on two factors: firstly, on the state of oxidation, or peroxide value of the fatty acid; and secondly, on the duration of contact of emulsion and enzyme. These effects are discussed in a subsequent section of this paper.

Enzyme inhibition by saturated fatty acids such as was found with the enzymes of group B is clearly unconnected with double bonds or their oxidation. It is an unspecific effect and possibly results from the affinity of the negative charges of the fatty acids for the positive charges on the protein. It has been previously discussed by Putnam[16] and Wills.[12]

On the other hand, enzymes of groups C and D are inhibited to a much greater extent by linoleic acid than by palmitic acid, and this effect must be directly related to the presence of unsaturated linkages or the peroxides formed at these bonds.

Bernheim *et al.*[7] have already shown that certain oxidative enzymes are inhibited by unsaturated fatty acids. It will be observed from Table 1 however, that the inhibitory effect of unsaturated fatty acids is not confined to oxidative enzymes, and that several enzymes of other classes, e.g. urease and glyoxalase are also inhibited.

A more significant property of the enzymes included in Groups C and D is that, with the exception of trypsin and cytochrome oxidase, they all possess essential —SH groups. Furthermore, all enzymes tested possessing essential —SH groups were inhibited by linoleic acid.

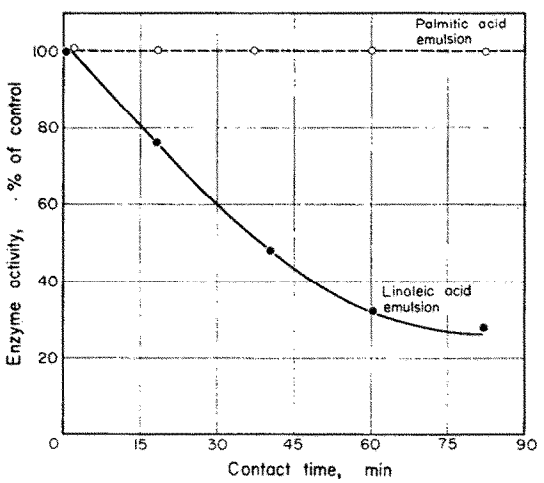

FIG. 1. A comparison of the effects of palmitic acid emulsion (4×10^{-4} M) and linoleic acid emulsion (4×10^{-4} M) on succinic oxidase 37 ° when the time of contact of emulsion and enzyme is varied.

Effect of contact time and temperature on enzyme inhibition

Most experiments described in this paper were carried out using a contact period of emulsion and enzyme of 20 min and at 37°. Inhibition of all enzymes of Groups C and D was found to increase with increase of the contact time. In Fig. 1 a comparison is made between the effects of palmitic and linoleic acids in equivalent concentrations

on succinic oxidase. It will be observed from Fig. 1 that inhibition of the enzyme increases progressively with time of contact.

For certain enzymes the temperature of the incubation was also found to be important. Thus the inhibition of urease by linseed acid emulsion was found to be much greater after 4 hr incubation at 37 ° than after 4 hr incubation at 16 °.

Relation between the extent of oxidation of the fatty acid emulsion and enzyme inhibition

Unsaturated fatty acids readily take up oxygen to give peroxides. These are probably hydroperoxides (—OOH) formed on a carbon atom adjacent to the double bond.[17]

Emulsions containing 10^{-2} M linoleic acid, linolenic acid or mixed linseed acids were oxidised by shaking in air at 37° for periods which were varied between 3 hr and 48 hr. Catalysts such as haemin which speed the rate of autoxidation were added to some emulsions. The extent of the oxidation was measured either (a) by a determination of the peroxide value by the ferric thiocyanate method[18] or (b) for emulsions of linolenic acid or mixed linseed acids by the thiobarbituric acid colorimetric method.[19]

A series of emulsions of unsaturated fatty acids of different peroxide values were tested on urease, succinic oxidase and papain. For each experiment the oxidised emulsion was incubated with the enzyme for 20 min at 37° before determination of the enzyme activity. Whichever method was used for the determination of the peroxidation, enzyme inhibition was always found to increase progressively in parallel with the increase of peroxide value of the emulsion. Results for urease and papain are shown in Figs. 2 and 3. Inhibition was found to be dependent on the time of contact

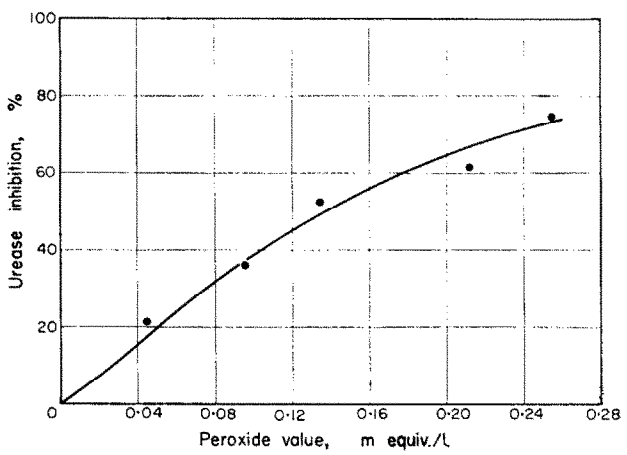

FIG. 2. Relation between the peroxide value of a mixed linseed acid emulsion (10^{-3} M) and urease inhibition. Peroxide values determined by the thiobarbituric acid method.

of emulsion and enzyme as in other experiments, but the fatty acid emulsion of higher peroxide value inhibited the enzyme much more strongly than an emulsion of low peroxide value when each was incubated with the enzyme for the same time period (Fig. 4).

Effect of ultraviolet light on mixtures of fatty acid emulsions and enzymes

Ultraviolet light catalyses the formation of peroxides from unsaturated fatty acids[20] and therefore irradiation of enzymes in presence of unsaturated fatty acids

would be expected to cause significant inactivation of enzymes sensitive to these peroxides.

In order to investigate the effect of ultraviolet light, open dishes of solutions were irradiated 30 cm from a Hanovia u.v. lamp. In each experiment the following systems were irradiated: (a) the enzyme alone, (b) the enzyme + palmitic acid emulsion (10^{-3} M or 4×10^{-4} M), (c) enzyme + linoleic acid or mixed linseed acid emulsion

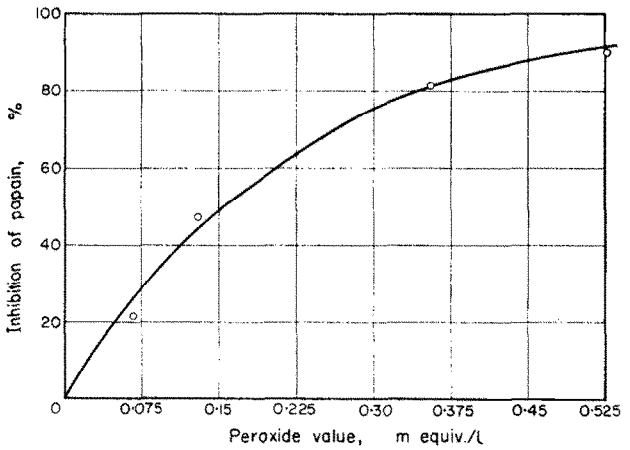

FIG. 3. Relation between the peroxide value of a linoleic acid emulsion (10^{-3} M) and inhibition of papain. Peroxide values determined by the ferric thiocyanate method.

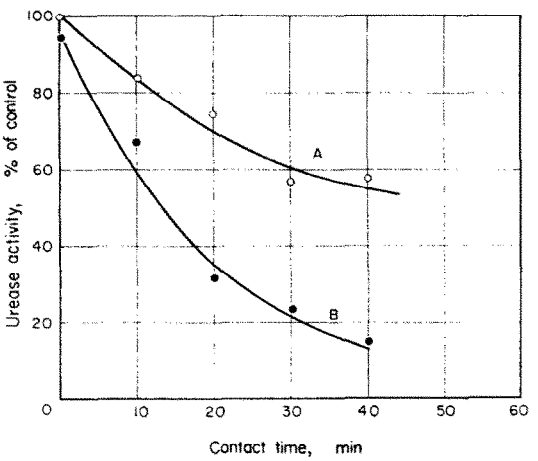

FIG. 4. Relation between the peroxide value of a linseed acid emulsion (10^{-3} M) and contact time on the inhibition of urease.
Curve A—Peroxide value = 0·045 mequiv/l.
Curve B—Peroxide value = 0·21 mequiv/l.

(10^{-3} M or 4×10^{-4} M), (d) linoleic acid or mixed linseed acid emulsions (10^{-3} M or 4×10^{-4} M), (e) palmitic acid emulsion (10^{-3} M or 4×10^{-4} M). After the irradiation the activity of the untreated enzyme was compared with the enzyme irradiated under conditions (a), (b) or (c). In addition, irradiated linoleic acid and irradiated palmitic acid emulsions were tested on the untreated enzyme.

Succinic oxidase, β-amylase, and invertase were irradiated under these conditions for periods which were varied between 2 hr and 6 hr. Succinic oxidase, after being treated with u.v. for 2 hr was 14 per cent inhibited, but the inhibition increased to 63 per cent if 4×10^{-4} M linoleic acid emulsion was present during the irradiation. Irradiated linoleic acid added to the untreated enzyme caused only 40 per cent inhibition after 30 min contact. Palmitic acid emulsion (4×10^{-4} M) on the other hand, did not alter the effect of u.v., the enzyme being 14 per cent inhibited as in absence of emulsion. The inhibition of β-amylase was increased from 4 per cent after 2 hr u.v. irradiation to 20 per cent when linoleic acid emulsion (10^{-3} M) was added before irradiation. On the other hand, invertase which had previously been shown not to be inhibited by fatty acid peroxides, was unaffected by irradiation in presence of 10^{-3} M unsaturated fatty acids.

Effect of lipoxidase

Lipoxidase catalyses the oxidation of unsaturated fatty acids such as linoleic or linolenic to form peroxides at their double bonds.

The effect of enzymically produced peroxides was investigated by incubating lipoxidase solution (0·5 mg/ml of a pure Sigma preparation) with $6{\cdot}6 \times 10^{-4}$ M linoleic acid emulsion for 20 min when oxygen uptake ceased. The resulting oxidised emulsion when tested on urease caused 77 per cent inhibition, whilst the untreated emulsion in the same concentration caused only 43 per cent inhibition. The effect of lipoxidase itself on urease was negligible. Inactivation of the urease was greater if it was incubated with lipoxidase actively oxidising the unsaturated fatty acids. Thus urease was 30 per cent inhibited by $6{\cdot}6 \times 10^{-4}$ M linseed acid emulsion added to the enzyme after oxidation was complete, but the inhibition was increased to 88 per cent if the lipoxidase oxidised the emulstion in presence of urease. Incubation of urease with lipoxidase alone for a 20-min period was found to have a negligible effect on urease activity.

Protection of enzymes against inhibition by unsaturated fatty acids

Experiments described in this paper have shown that enzymes possessing —SH groups are most sensitive to inhibition by peroxides. If the —SH groups are vulnerable sites for enzymic inactivation by peroxides, protection is likely to be afforded and the inhibited enzymes possibly reactivated by the addition of sulphydryl compounds such as cysteine or glutathione.

TABLE 3. EFFECT OF CYSTEINE ON THE INHIBITION OF UREASE BY LINSEED ACID EMULSION

Time of contact before cysteine addition (min)	Inhibition (per cent)
No cysteine	69
0	25
5	33
30	67

Cysteine (10^{-2} M) added after urease had been in contact with linseed acid emulsion ($3{\cdot}3 \times 10^{-3}$ M) for time specified above.

The effect of cysteine (10^{-2} M) was examined, either by adding it to the urease before addition of the fatty acid emulsion ($3{\cdot}3 \times 10^{-3}$ M linseed acid emulsion) or by adding it a short period after. Results are shown in Table 3. As will be seen from this

table, cysteine gave partial protection of the enzyme and partial reactivation if the period of contact of emulsion and enzyme was not prolonged. After longer contact periods no reversal was possible.

Cysteine (10^{-2} M) was also found to protect succinic oxidase, reducing the inhibition by $3{\cdot}3 \times 10^{-4}$ M mixed linseed acids from 80 per cent to 36 per cent. Papain was also protected, 10^{-2} M cysteine reducing the inhibition by 10^{-3} M mixed linseed acid emulsion from 65 per cent to 12 per cent.

DISCUSSION

The inhibition of enzymes by fatty acids described in this work clearly falls into two categories: (i) that which is unspecific, being caused by saturated and unsaturated fatty acids alike, and (ii) that which is specifically caused by unsaturated fatty acids.

Enzyme inhibition of type (i) has been previously described by Peck[21] who found that trypsin was inhibited by sodium salts of saturated fatty acids and by Wills[12] who found that the related anionic detergents such as sodium dodecyl sulphate powerfully inhibited a large variety of enzymes. Many of the enzymes tested were strongly inhibited by anionic detergents when the pH of the solution was acid. This inhibition is probably the result of combination of the basic groups of the protein with the acidic detergent groups. So far as possible, this effect was avoided in the present work by using a pH of 7·4 for most experiments.

In view of these findings it may be concluded that inhibition of enzymes of group B (enzymes which are inhibited by saturated and unsaturated fatty acids to an approximately equal extent) is probably a non-specific effect resulting from the combination of negatively charged fatty acids and basic protein groups which leads to the denaturation of the enzyme.

The inhibition of enzymes of group D by unsaturated fatty acids is likely to be the result of two distinct effects. Firstly, a result of combination of the negative charged fatty acids with the basic protein groups, and secondly, a result of the presence of unsaturated bonds in the fatty acid. However, when the inhibitory effects of less concentrated emulsions (e.g. 4×10^{-4} M) of saturated and unsaturated fatty acids were compared it was frequently found that only unsaturated fatty acids caused inhibition (Fig. 1).

Enzymes of group C are not affected at pH 7·4 by negative charges on the fatty acids and inhibition in these cases is solely a result of the presence of double bonds or peroxides in the unsaturated fatty acids.

When first prepared, emulsions of unsaturated fatty acids gave small but measurable peroxide values. Such emulsions caused some enzyme inactivation but this was found to increase progressively as the period of incubation of the emulsion with the enzyme was increased.

When unsaturated fatty acids are oxidised, peroxides, probably hydroperoxides, are formed at the CH groups adjacent to the double bonds, a seift of o double bond occurring during the oxidation.[17]

Inhibition of the enzymes of groups C and D by unsaturated fatty acids may be explained on the basis that it is these peroxides which are responsible. Evidence for this opinion is summarised in Figs. 2 and 3, where it is shown that the enzyme inhibition increases with increase of peroxide value. Emulsions of low peroxide value were found to be much less inhibitory than emulsions of higher value but the inhibition increased

with the time of contact with the enzyme. This was presumably the result of a slow development of peroxide. When peroxides were preformed the inhibitory effect was developed much more rapidly (Fig. 4). Experiments using lipoxidase and ultraviolet light showed that these peroxides were often more toxic if formed in presence of the enzyme than if added to the enzyme after formation. The reason for this is not clear but it is possible that free radicals generated during peroxide formation are more toxic to the enzyme than the peroxides themselves. This phenomenon may be of considerable importance in relation to possible *in vivo* actions of peroxides which are discussed later.

An inspection of the enzymes listed in Table 1 will indicate that —SH enzymes are most readily inactivated by linoleic acid. In fact, all enzymes tested which are known to possess essential —SH groups were inactivated by linoleic acid, and trypsin and cytochrome oxidase are the only enzymes of groups C and D which are not normally regarded as —SH enzymes. The —SH groups of enzymes are likely to be vital targets for peroxide inhibition on account of (a), the nature of the enzymes inhibited and (b) the fact that —SH compounds such as cysteine or glutathione are protective. After short periods of contact, limited reversal of inactivated sulphydryl enzymes by —SH compounds was obtained but after longer contact periods, no reversal was possible. Furthermore, it has been previously shown by Dubouloz and Fondarai[8] that the —SH groups of sulphydryl proteins are oxidised to —S—S— and further oxidation products by fatty acid peroxides. The results of the experiments using —SH enzymes may be interpreted as indicating that there is an initial oxidation of —SH to —S—S— but that further changes are produced in the molecule. Simple oxidation of —SH to —S—S— cannot be the only reaction involved since this would be reversed by cysteine or glutathione. In this respect, the inactivation of sulphydryl enzymes by fatty acid peroxides is closely parallel to inactivation of this class of enzyme by organic peroxides as described by Wills.[15]

These experiments have confirmed the observations of Bernheim *et al.*[7] that oxidation enzymes are inhibited but it has been found that the inhibitory effect is not confined to this class of enzyme, since several other classes of enzyme, e.g. hydrolases are inhibited. As discussed above, it is considered that the most sensitive enzymes are —SH and the fact that several are oxidative is incidental. All the oxidative enzymes found to be inhibited by Bernheim *et al.*[7] are normally regarded as —SH enzymes. Tappel and Zalkin[9] have also shown recently that mitochondria took up oxygen forming peroxides, several oxidative enzymes being destroyed in the process.

The true role of the inhibitory effect of peroxides of fatty acids *in vivo* is not easy to establish, but nevertheless, it is possible that it may be of considerable importance in metabolic control. The oxidation of fatty acids to peroxides can be powerfully catalysed by iron compounds such as haemin in very low concentrations.[22] These peroxides may then slow a vital metabolic path by inhibiting a vital —SH enzyme. Located in important membranes around or within the cell, probably as constituents of phosphatides, it appears possible that unsaturated fatty acids may be well sited to exert an important metabolic control.

It is also possible that the oxidation of unsaturated fatty acids may be connected with the effects of radiation. Relationships between peroxides and radiation have been established from several avenues of research; firstly, it has been shown that peroxides are formed in irradiated animals,[4] secondly, that some of the toxic effects of injected