be advanced for the presence of trans-trans conjugation in spots C₁ and C₁A₁, although this mechanism does not afford a reason for the difference in RF value between spots B and C₁, C₁A₁.

The alternative explanation, that the dimeric material is formed by a Diels–Alder addition (Fig. 2) appears to be excluded on several counts. If dimerization occurred by this mechanism, absorption due to conjugated dienes would not be expected, and indeed the dimeric fraction from co-polymerization of 9, 11 and 9, 12 methyl octadecadienoate at 300° in an inert atmosphere (which might be expected to cause polymerization by a Diels–Alder mechanism) showed only a single band for trans-absorption at 10.3 μm. Moreover, we were unable to aromatize spot B from deodorized CSO either by the bromination–dehydrobromination procedure of Clingman, Rivett & Sutton (1954) or by heating to 275° with palladium-on-charcoal catalyst, a method used by Paschke, Peterson & Wheeler (1964) to demonstrate the presence of a cyclohexene structure in a Diels–Alder type dimer.

Finally, chromatograms of the UNA of crude groundnut oil, and groundnut oil in the various stages of refining, all showed the presence of spot B. The maximum temperature of 180° reached in the refining sequence is lower than that required for Diels–Alder addition of fatty acids, and dimer formation is much more likely to have occurred by an oxidative mechanism.

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REFERENCES


EXPLANATION OF PLATES


The effect of lipid peroxides on the biochemical constituents of the cell

By P. J. O'BRIEN and A. C. FRAZER, Department of Medical Biochemistry and Pharmacology, University of Birmingham

Many food lipids can oxidize rapidly in the presence of oxygen or peroxides and catalysts, such as ionizing radiations, light, lipoxidases, or metals. Antioxidants in
food tend to keep oxidation to a minimum, but processing, refining, preservation and storage of food may alter the relationships between antioxidant and oxidant, so that significant oxidation may occur. In studying the possible toxicological effects of the numerous products of oxidation, many mixtures of, or fractions derived from, oxidized materials have been studied. There is little doubt that one of the most reactive products of fat oxidation is lipid hydroperoxide. The studies described in this paper were carried out to provide a clearer picture of the possible effects lipid hydroperoxides might have on the body and the means by which the body may protect itself against them.

_Destruction of lipid hydroperoxides in the gastro-intestinal tract and in other tissues_

Using [1-14C]linoleic acid hydroperoxide, Freeman (1964) showed that trace amounts given orally to rats were rapidly destroyed in the gastro-intestinal tract and the products absorbed. Glavind & Tryding (1960) could find no peroxide in lymph in rats given oxidized olive oil. As lymph, bile, or pancreatic juice did not have any significant effect on the peroxide group and as haemo-proteins are effective in catalysing the decomposition of lipid peroxides (Maier & Tappel, 1959) they would seem to be destroyed either by reaction with other food materials or by the intestinal mucosa.

Nishida & Kummerow (1959–60) noted delayed fat absorption in animals fed on fats containing peroxide. Olcott & Dolev (1964) gave oxidized methyl linoleate to rats and found that a level of 800 μmoles/100 g caused diarrhoea and at 1600 μmoles/100 g death occurred. When the peroxide was injected intraperitoneally 150–160 μmoles/100 g were lethal. However, post-mortem examination studies on similar experiments with mice carried out in this laboratory indicated that death was due to peritonitis and that intramuscular injections caused no deaths, although the lipid peroxides were absorbed. Furthermore, Freeman (1964) showed that more than 90% of [1-14C]linoleic acid hydroperoxide injected intramuscularly was degraded before incorporation into liver lipids. It appears, therefore, that decomposition of lipid peroxides can occur in many tissues. Incubation of linoleic acid hydroperoxide with subcellular fractions showed that lipid hydroperoxides can be degraded in the liver (O’Brien, 1964).

If the mucosa of the small intestine is a major site of inactivation it is possible that the mucosa suffers damage in the process. Under normal circumstances this may not be serious, since the intestinal epithelium is completely replaced every 24–48 h. If, however, regeneration of the mucosa is slower than normal, damage of this nature might lead to more extensive tissue damage. If the mucosal barrier becomes ineffective, for this or any other reason, even greater penetration of hydroperoxides into the body might occur. This aspect of the effect of lipid hydroperoxides is under investigation in our laboratories at the present time.

There is also evidence suggesting that in vivo peroxidation of lipids may occur in tissues and can lead to a variety of signs of vitamin E deficiency (Tappel, 1962). However, the methods available for determination of peroxides in tissues are unsatisfactory, and it is difficult to be certain that peroxides have not been formed.
in the tissues after removal from the body. Present evidence indicates that any endo-
genous peroxide pool is likely to be extremely small. Our studies are only concerned
with the effect of peroxides added to cells or cellular components. Thus, a lipid hydro-
peroxide has been prepared, isolated and purified and its effect on mitochondrial
respiration investigated.

**Effect of linoleic acid hydroperoxide and other fatty acids on succinate oxidation by
mitochondria**

Mitochondria were prepared from rat livers by the differential centrifugation
method of Schneider (1948). Linoleic acid hydroperoxide was prepared by direct air
oxidation of linoleic acid, and isolated from the oxidation mixture by partition
between light petroleum and 75% aqueous methanol. The isolated hydroperoxide
was estimated by U.V. spectrophotometry and thin-layer chromatography to be
95–99% pure.

The effect of linoleic acid hydroperoxide on the oxygen uptake by mitochondria
with and without succinate present was measured. The hydroperoxide was found to
be effective at $10^{-8}$M in inhibiting the oxidation of succinate by 4 mg of mito-
chondria. Free fatty acids, owing to their surface-active properties, have a marked
effect on mitochondrial metabolism; similar experiments with oleic and linoleic acids
were, therefore, carried out. Oleic and linoleic acid were each found to be as effective
as hydroperoxide in inhibiting succinate oxidation. It was impossible to say whether
the powerful hydroperoxide moiety caused any of the observed inhibition although,
because of its greater solubility in water, linoleic acid hydroperoxide is not as strong
a surface-active agent as linoleic acid or oleic acid. However, the results of experi-
ments with the addition of cytochrome $c$ to the incubation medium indicated that
cytochrome $c$ offered better protection against the hydroperoxide than against oleic
or linoleic acids. The added cytochrome may be replacing cytochrome $c$ damaged
in the mitochondria, or it may be decomposing the hydroperoxide into less surface-
active substances.

**Effect of hydroperoxide on mitochondrial and microsomal cytochromes**

The absorption spectrum of mitochondria brought into solution with sodium
lauryl sulphate was examined before and after adding hydroperoxide. These experi-
ments indicated that hydroperoxide caused a rapid disappearance of the cytochrome
Soret peak.

In the first series of experiments, the poor resolution of the Soret peak did not
enable any quantitative results to be obtained. However, similar experiments using
microsomal fractions were much more clear-cut, and from a calculation of the cyto-
chrome content it was estimated that 50 molar equivalents of hydroperoxide were
required to decrease the Soret peak by 50% in 1 min at 20°. These results indicated
that cytochrome $b_{5}$ and possibly also the CO-binding pigment in microsomes were
destroyed by hydroperoxide. Addition of hydrogen peroxide instead of hydro-
peroxide had no effect on the Soret peak of the mitochondrial or microsomal cyto-
chromes. It was concluded that catalase, present in the mitochondrial and micro-
somal preparation, rapidly decomposed the hydrogen peroxide into molecular O₂ before it could interact with the cytochromes.

**Effect of hydroperoxide on the ability of cytochrome c to restore succinate oxidation in extracted mitochondria**

Bound cytochrome c was extracted from rat-liver mitochondria by the procedure of Jacobs & Sanadi (1960). Fig. 1 shows the restoration of succinate oxidation by

![Graph](https://www.cambridge.org/core/images/fig1.png)

**Fig. 1.** The restoration of succinate oxidation of extracted mitochondria by cytochrome c. •, cytochrome c only; ○, cytochrome c pre-incubated with 17 equivalents of linoleic acid hydroperoxide; △, cytochrome c pre-incubated with 50 equivalents of linoleic acid hydroperoxide.

added cytochrome c for a mitochondrial preparation. The initial rate of oxygen uptake was calculated from the first 20 min of incubation. Pre-incubation of the cytochrome c solution with hydroperoxide markedly reduced the ability of cytochrome c to restore succinate oxidation. The same results were obtained when treated and untreated cytochrome c were adjusted to pH 4 and extracted with diethyl ether (to remove hydroperoxide products). A pre-incubation time of 1 min was necessary for maximal effect.

Jacobs & Sanadi (1960) found that the depleted mitochondria took up the same amount of cytochrome c as had been previously extracted and that the restoration of succinate oxidation and oxidative phosphorylation by the added cytochrome c was complete. It was therefore likely that the added cytochrome c was bound at the same sites as those occupied before extraction. The lipid hydroperoxide either altered the
cytochrome c so that it could no longer be bound at the mitochondrial sites or damaged it so that it could not function normally, even though bound. The latter explanation seems less likely, as from Fig. 1 it can be seen that increasing the concentration of treated cytochrome c increased the restoration of succinate oxidation.

**Interaction of hydroperoxide with cytochrome c**

The addition of hydroperoxide to a solution of cytochrome c caused an immediate decrease in the Soret peak at 409 nm, followed by a slower decrease exponential with time. The total decrease was found to be directly proportional to the concentration of hydroperoxide, and independent of the concentration of cytochrome c. At pH 8.5, 35 molar equivalents were required to decrease the Soret peak by 50%. The rate of this decrease was, however, dependent on the concentration of cytochrome c. With $0.9 \times 10^{-6}$M cytochrome c half of the total decrease occurred within 1 min at 20°. At pH 7.0 the reaction was nearly complete within 1 min at 20°.

The addition of cytochrome c to a solution of hydroperoxide caused a rapid decomposition of hydroperoxide as measured by the optical density at 233 nm. This decrease was exponential with time. The products showed diene conjugation (233 nm) and triene conjugation (284 nm).

Cyanide and azide at $0.1 \text{m}$ completely inhibited the decomposition of hydroperoxide and the decrease in Soret peak. It is concluded that the complexes formed between cyanide and azide and the central Fe atom are ineffective as catalysts in the decomposition of the lipid hydroperoxide. The decrease of the Soret peak would seem to be the result of interactions with free radicals produced by the decomposition of the hydroperoxide. In turn, the damage to cytochrome c decreases its effectiveness as a catalyst in the decomposition of hydroperoxide, so that the reaction rate is markedly exponential with time.

Fig. 2 shows the effects of increasing amounts of hydroperoxide on the absorption spectrum of cytochrome c. Two stages were discerned: Stage (1). The regions 346–457 nm, 512–568 nm, showed a decrease in optical density with maximal decrease at 406 nm (i.e. the Soret peak) and at about 531 nm. The regions 457–512 nm and 568–700 nm showed an increase in optical density, with maximal increase at 470–490 nm. Stage (2). This stage began when the Soret peak had decreased in optical density by 40%. The region 457–512 nm now began decreasing. Thus, the whole region 346–570 nm decreased in optical density. The region 580–700 nm, however, showed no change, until eventually the region 350–700 nm was continuous, with no peaks and slowly decreasing. The former bright red solution was now a very pale green. In stage 2 it is likely that the porphyrin ring has been opened by the oxidation. Addition of dithionite to cytochrome c after interaction with the hydroperoxide showed that the $\alpha$ and $\beta$ bands decreased linearly during stages 1 and 2. The total decrease in the $\alpha$, $\beta$ and Soret bands was directly proportional to the amount of hydroperoxide present.

The effects of lipid hydroperoxide on the absorption spectrum of reduced cytochrome c at pH 8.5 showed that the addition of only one equivalent of lipid hydroperoxide caused the instantaneous oxidation of reduced cytochrome c to oxidized
cytochrome c. Under similar conditions, hydrogen peroxide oxidized reduced cytochrome c very slowly. Thus, 6 equivalents of hydrogen peroxide took 40 min to oxidize reduced cytochrome c.

**The effects of other peroxides on cytochrome c**

The results obtained on the rate of decrease of the Soret peak of cytochrome c by different peroxides are shown in Table 1. Linoleic acid hydroperoxide was found

![Absorption spectrum of cytochrome c after reaction with increasing amounts of linoleic acid hydroperoxide.](https://www.cambridge.org/core/terms). https://doi.org/10.1079/PNS19660005

Table 1. The effects of peroxides on cytochrome c

<table>
<thead>
<tr>
<th>Peroxide</th>
<th>pH 7</th>
<th>pH 8.5</th>
<th>50% ethanol, pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic acid hydroperoxide</td>
<td>0.12</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>20.0</td>
<td>64.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Succinoyl peroxide</td>
<td>0.23</td>
<td>No effect</td>
<td>0.6</td>
</tr>
<tr>
<td>Cumene hydroperoxide</td>
<td>5.0</td>
<td>5.0</td>
<td>12.0</td>
</tr>
<tr>
<td>t-Butyl hydroperoxide</td>
<td>—</td>
<td>—</td>
<td>33.0</td>
</tr>
<tr>
<td>Benzoyl peroxide</td>
<td>—</td>
<td>—</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Peroxide added to reaction mixture containing 16.5 nm-moles cytochrome c, 100 μmoles tris-HCl buffer, 2.5 ml water or 50% ethanol at 20°.
to be most effective (at pH 8.5 it had 400 times the effectiveness of hydrogen peroxide). At pH 8.5 some of this effect may be due to its surface-active properties, which would enable it to open up the cytochrome by hydrophobic forces between the long hydrocarbon chains of the fatty acid and the hydrophobic amino acid side-chains, resulting in exposure of the haem group to the hydroperoxide.

Because of the insolubility in water of t-butyl hydroperoxide and benzoyl peroxide, these peroxides were compared with the other peroxides in 50% ethanol. In this medium cytochrome c appeared to have a much more open structure, as hydrogen peroxide was now very effective. However, on this basis it was surprising that some of the organic peroxides were less effective.

The results in Table I do not represent initial rates of reaction, as with linoleic acid hydroperoxide and succinyl peroxide these were too fast to measure. Therefore, in the comparison between the peroxides, the figures in Table I tend to underestimate the rate at which cytochrome c was decomposed by these two peroxides. The effectiveness of peroxides in altering cytochrome c may be related to their ease of catalytic decomposition. Linoleic acid hydroperoxide and succinyl peroxide may be decomposed by cytochrome c much more rapidly than the other peroxides investigated.

**The separation of the products of cytochrome c after reaction with hydroperoxide**

Cytochrome c was reacted with 30 equivalents of hydroperoxide at pH 8.0, so that the Soret peak (at 409 nm) of cytochrome c had decreased in intensity by 50%. This solution was then applied to the top of a Sephadex G 100 (Pharmacia, Uppsala, Sweden) column, being eluted with 0.05 M tris-hydrochloride buffer (pH 8.0, containing KCl (0.1 M)). A diffuse green band soon separated from the red band of cytochrome c. As Sephadex-gel filtration separates proteins mainly according to their molecular weights, the green band was considered to be polymerized cytochrome c. Furthermore, a determination of the void volume using blue dextran indicated that a large part of the polymer fraction was completely excluded from the internal cavities of the gel, and thus would have a molecular weight of at least 100,000 (that is, over 8 cytochrome units). A spectrophotometric examination of the column effluent fractions revealed that 30–40% were present as the monomer. An accurate determination by this method was not possible, owing to the presence of considerable amounts of hydroperoxide degradation products in the polymeric fractions. These products were mainly in the first fractions, and this may indicate that the more polymerized the cytochrome c products became the more hydroperoxide degradation products were associated with them. On adjusting these fractions to pH 3, 85–90% of the hydroperoxide degradation products, as judged by ultraviolet absorption of the fractions, were easily extracted by shaking with diethyl ether. This suggested that the hydroperoxide products might be associated with cytochrome c by the same types of bonds as those by which fatty acids are bound to proteins, i.e. electrostatic and hydrophobic bonds. The ultraviolet absorption spectrums of the extracts showed diene and triene conjugation.
In order to correlate the decrease in the Soret peak with the degree of polymerization of cytochrome $c$, the reaction of hydrogen peroxide with cytochrome $c$ was studied, so that spectrophotometry could be used to analyse the fractions. Fig. 3 shows the results of four experiments in which increasing amounts of hydrogen peroxide were incubated with cytochrome $c$ for 24 h. The protein content of each fraction was measured from the absorption at 215 nm. Although hydrogen peroxide reacted much more slowly than lipid hydroperoxide, after 24 h only 12 equivalents of hydrogen peroxide were needed to decrease the Soret peak of a $3.7 \times 10^{-4} M$ solution of cytochrome $c$ by 50%. From Fig. 3 it can be calculated that 30–35% of cytochrome $c$ was still present as the monomer. The lack of separation of the polymeric cytochrome $c$ into dimers, trimers, tetramers, and higher polymers, may be due to the complexity and different degrees of denaturation of the protein products. From Fig. 3 the monomer can be shown to display a Soret peak (409 nm) 70% of that of unchanged cytochrome $c$. The polymerized cytochrome $c$ fraction shows a Soret peak 30% of unchanged cytochrome $c$. The observation that a decrease in Soret peak

![Figure 3](https://www.cambridge.org/core/fig/97d6e4e5468c492d96f6f0a47056c247)
can occur without polymerization suggests that polymerization may occur immediately after the decrease in Soret peak. Further proof that the decrease in Soret peak is not a direct result of the polymerization reaction was obtained when cytochrome c which had been reacted with enough hydrogen peroxide or lipid hydroperoxide at pH 2.6 to reduce the Soret peak by 50% was chromatographed on Sephadex. 90% of the cytochrome c was found to be present as the monomer with a Soret peak 50% of that of unreacted cytochrome c.

Although the cytochrome c products formed in the interaction with hydrogen peroxide had very similar spectrums to those formed with linoleic acid hydroperoxide, thus indicating similar products, they were much more soluble in water. When sufficient hydroperoxide was added to decrease the Soret peak of a solution of $4 \times 10^{-4}$M cytochrome c by 50%, precipitation of cytochrome c occurred in the pH range 3.5-8.0. No precipitation occurred when hydrogen peroxide was used instead of hydroperoxide. Desai & Tappel (1963) found that oxidized linolenic acid could form co-valent bonds with the cytochrome c, and they may account for lowered solubility. Alternatively, hydroperoxide may polymerize cytochrome c to a greater extent.

**Discussion**

These studies show that a lipid hydroperoxide differs greatly from hydrogen peroxide in its effects on cellular constituents. In the first place it is not rapidly destroyed by a catalase mechanism, so that this protective mechanism is ineffective against linoleic acid hydroperoxide. In addition to effects that may be due to the fatty acid character of the lipid hydroperoxide molecule, lipid hydroperoxide has a striking effect on cytochrome c of the cell. This effect is due to its hydroperoxide moiety. It causes damage to the haematin ring and polymerization of the protein. After interaction with lipid hydroperoxide, cytochrome c can no longer function efficiently in the mitochondrial electron transport system. It seems likely that these effects of the lipid hydroperoxide may be due to free radicals released by decomposition.

Other interactions between lipid peroxides and proteins have been described. Nishida & Kummerow (1959–60) have described association of plasma $\beta$-lipo-proteins induced by methyl linoleate hydroperoxide. Polymerization products, associated with tissue ageing, may also arise from effects of peroxides (Hendley, Mildvan, Reporter & Strehler, 1963). Tappel (1962) has reported inactivation of mitochondrial enzymes, damage to the electron transport chain and the release of hydrolases from lysosomes as a result of in vitro peroxidation. Hochstein & Ernster (1964) have demonstrated inactivation of enzymes associated with the membranes of the endoplasmic reticulum by peroxides.

It may, therefore, be concluded that, if linoleic acid hydroperoxide should find its way into cells, it will cause severe damage to cytochrome c and bring about significant interference with the functional efficiency of mitochondria.

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Animal diseases associated with autoxidation of dietary fat

By C. A. Grant, Department of Pathology, Karolinska Hospital, Stockholm 60, Sweden

The theme of this Symposium—‘Nutritional and toxicity problems associated with fats’—is an important one for nutritionists and biochemists. It is also an important one for pathologists who encounter these problems in a particularly tangible form, as part of everyday work in diagnosing animal diseases and in the evaluation of responses of animals to experimental procedures.

The important naturally occurring animal diseases associated with dietary fat are those associated with autoxidation of dietary fat. Pigs present the major problems although other species of domestic animals and poultry as well as wild animals in captivity are involved at times. The position of young ruminants, lambs and calves, is ambiguous, as Blaxter (1962) has pointed out.

Dietary background

Our present and still fragmentary knowledge of the relation between naturally occurring animal diseases and autoxidation of dietary fat stems from what were at first laboratory curiosities in chicks—encephalomalacia (Pappenheimer & Goettisch, 1931) and ‘exudative diathesis’ (Dam & Glavind, 1939). Dam and his associates in the 1930’s and 1940’s established a connexion between these lesions, oxidative instability of unsaturated dietary fat, and tocopherol. The spectrum of what were originally experimental lesions has widened to include skeletal muscle degeneration, dietetic liver necrosis, ‘yellow fat’ and haemolysis as well as the original observations of sterility and foetal resorption, in a number of species of laboratory animals. On the dietary side of the equation, the selenium-containing Factor 3 of Schwarz has come into the picture (Schwarz & Folz, 1957).

The classical experimental diets used to induce these lesions included large amounts of unsaturated fat, usually cod-liver oil. During the 1950’s, the diet based