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# Lipid Peroxide Formation in Microsomes

## GENERAL CONSIDERATIONS

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1. Liver microsomes form lipid peroxide when incubated with ascorbate or NADPH, but not with NADH. Increasing the concentration of ascorbate beyond the optimum (0·5mM) decreases the rate of lipid peroxide formation, but this effect does not occur with NADPH. Other reducing agents such as *p*-phenylenediamine or ferricyanide were not able to replace ascorbate and induce lipid peroxide formation. 2. The rate of ascorbate-induced peroxidation is optimum at pH6·0 whereas the rate of the NADPH system is optimum at pH7·0. Both systems require phosphate for maximum activity. 3. Lipid peroxide formation occurs at the maximum specific rate in very dilute microsome suspensions (0·15mg. of protein/ml.). 4. Treatment of microsomes with deoxycholate and other detergents causes membrane disintegration and inhibits lipid peroxide formation. 5. Lipid peroxide formation is accompanied by a rapid uptake of oxygen and there is a large excess of oxygen utilized for each molecule of malonaldehyde measured in the peroxide method. 6. Boiled microsomes form lipid peroxide in the presence of ascorbate, but not if NADPH is added. 7. Lipid peroxide formation induced by NADPH is strongly inhibited by *p*-chloromercuribenzoate, weakly inhibited by *N*-ethylmaleimide and unaffected by iodoacetamide. Ascorbate-induced peroxidation in untreated microsomes is unaffected by *p*-chloromercuribenzoate, but inhibited if boiled microsomes are used. These experiments may be interpreted on the basis that a ferredoxin-type protein forms part of the system in which NADPH induces lipid peroxide formation. 8. Most heavy-metal ions, with the exception of inorganic iron ($Fe^{2+}$ or $Fe^{3+}$), which activates, inhibit both ascorbate-induced and NADPH-induced peroxidation. $Mg^{2+}$ increases the rate of peroxidation whereas $Ca^{2+}$ inhibits it. 9. Lipid peroxide formation is inhibited strongly by GSH and weakly by cysteine. Ascorbate-induced peroxidation is much more sensitive than NADPH-induced peroxidation. 10. Peroxidation is strongly inhibited by addition of low concentrations (0·01–0·1mM) of cytochrome *c* or of haemoglobin. 11. It is considered that lipid peroxide formation occurs as a result of the operation of the microsomal electron-transport chain switching from hydroxylation to oxidize unsaturated lipids of the endoplasmic reticulum.

The fact that lipid peroxides, as measured by the thiobarbituric acid method, are formed in homogenates of many different tissues after incubation, has been known for a number of years (Bieri & Anderson, 1960; Zalkin & Tappel, 1960). Separated nuclei, mitochondria, lysosomes and microsomes of liver all form lipid peroxides after incubation, but the quantity and rate of lipid peroxide formed in the microsomal fraction is much greater than in other fractions. This difference is even more marked after subjecting the particles to ionizing radiation (Wills & Wilkinson, 1967). Tissue homogenates and subcellular fractions also catalyse the peroxidation of added unsaturated fatty acids such as linoleic acid or linolenic acid (Wills, 1966).

Iron, either in an inorganic form or as a haem complex, is likely to form an important part of the catalytic system in tissue homogenates (Wills, 1966), and lipid peroxidation in the mitochondrial fraction is stimulated by addition of iron (Hunter *et al.* 1964). Ascorbate stimulates peroxide formation in tissue homogenates (Wolfson, Wilbur & Bernheim, 1956) and in mitochondrial suspensions (Ottolenghi, 1959; Hunter *et al.* 1964) and is inhibited by GSH (Christophersen, 1968). Microsomal peroxidation is stimulated by ascorbate or NADPH (Hochstein & Ernster, 1963), and is accompanied by the disappearance of some polyunsaturated fatty acids from the phospholipids (May & McCay, 1968).

Lipid peroxides may be important as a factor causing, or a stage in, general membrane damage and they have been implicated in damage to mitochondrial membrane (Hunter *et al.* 1964), the erythrocyte membrane (Tsen & Collier, 1960), the lysosome membrane (Wills & Wilkinson, 1966) and the endoplasmic reticulum of liver cells by carbon tetrachloride (Recknagel & Ghoshal, 1965).

In view of the capacity of the microsomal fraction to form large quantities of lipid peroxide, and because peroxide formation may be of importance in the overall metabolism of the endoplasmic reticulum or its destruction *in vivo*, a detailed study has been made of the factors affecting, and possible mechanisms of, the process in the microsomal fraction. Special attention has been paid to NADPH-induced peroxidation as this may be linked to microsomal hydroxylation (Wills, 1969*b*). A preliminary account of this work has already been published (Wills, 1967).

## EXPERIMENTAL

*Materials.* $NADP^+$, sodium isocitrate and *p*-chloromercuribenzoic acid were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), thiobarbituric acid and malonaldehyde tetraethylacetal from Kodak Ltd. (Kirby, Lancs.), *N*-ethylmaleimide from Mann Research Laboratories (New York, N.Y., U.S.A.) and haemoglobin, haemin and cytochrome *c* from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). All other chemicals were purchased from British Drug Houses Ltd. (Poole, Dorset).

*Buffer solutions.* These were prepared as described by Dawson, Elliott, Elliott & Jones (1959). Tris–HCl or phosphate buffers were used in most experiments.

*Microsomes.* The microsomal fraction, henceforth described as 'microsomes', was prepared from the livers of 6–10-month-old male Wistar rats. Livers were homogenized in 0·25 M-sucrose and, after removal of nuclei, mitochondria and lysosomes by following the procedure of Aldridge (1957), the microsomal suspension was centrifuged at 100000 *g* for 1 hr. as described by Wills & Wilkinson (1967). After the supernatant had been removed completely the sedimented microsomes were usually suspended in 125 mM-KCl, (1 ml. of solution/g. of liver used for the preparation). This suspension is termed 100% microsomal suspension and contained 15·0 ± 1·49 mg. of protein/ml. (determined colorimetrically by the method of Lowry, Rosebrough, Farr & Randall, 1951). For several experiments the sedimented microsomes were washed either with 0·25 M-sucrose or with 125 mM-KCl and centrifuged again for 1 hr. at 100000 *g* before suspension in KCl.

Microsomes were also suspended in other media and in other concentrations as described in the text. All experiments described were carried out immediately after preparation of the microsomal suspension.

*Microsomal fractions.* These were prepared by a method based on that of Dallner (1963). Microsomes prepared as described and suspended in KCl were treated with 0·1% (w/v) sodium deoxycholate with agitation for 2 min. at 0° and then centrifuged again at 100000 *g* for 30 min. Three fractions were obtained: a sediment composed mainly of ribosomes, a fluffy layer above the sediment containing membranes of the endoplasmic reticulum and the supernatant. The supernatant was removed and the fluffy layer and sediment were resuspended in 125 mM-KCl (1 ml./g. of liver used for the preparation).

*Lipid peroxide.* This was determined by the thiobarbituric acid method of Wilbur, Bernheim & Shapiro (1949), as modified by Wills & Wilkinson (1967) with 0·5 ml. or 1·0 ml. of diluted microsomes. The method was calibrated with pure malonaldehyde and the molar extinction coefficient quoted by Sinnhuber, Yu & Yu (1958), $\epsilon_{530}$ $1{\cdot}56 \times 10^5$ cm.$^2$ m-mole$^{-1}$, was confirmed and used in all calculations for expression of the extent of peroxidation.

*NADPH.* This was prepared by enzymic reduction of $NADP^+$. The incubation mixture contained $NADP^+$ (0·54 mM), tris–HCl buffer, pH 7·0 (20 mM), sodium isocitrate (10 mM) and isocitrate dehydrogenase (Sigma type IV) (0·05 ml.) in a total volume of 5·0 ml. The mixture was incubated at 37° for 30 min., boiled for 0·5 min. to destroy the enzyme and the concentration of NADPH determined by measuring $E_{340}$ and using $6{\cdot}22 \times 10^3$ cm.$^2$ m-mole$^{-1}$ as the molar extinction coefficient of NADPH. The concentration of NADPH in most experiments was 40 $\mu$M.

*Lipid peroxidation studies.* The rate of formation of lipid peroxide was studied by incubating the microsomal suspension in a medium containing phosphate buffer, usually pH 7·0 (20 mM), NADPH (40 $\mu$M), nicotinamide (20 mM) and KCl (90 mM) at 37°. In most experiments the concentration of microsomal suspension was 10% or 20% (1·5 mg. or 3·0 mg. of protein/ml.), but other concentrations were used as described in the Results section. Samples of incubation mixture (0·5 ml.) were taken at intervals for determination of the rate of lipid peroxide formation by the thiobarbituric acid method during a total incubation period of 15 min. For the study of ascorbate-induced peroxidation, ascorbate (0·5 mM) replaced the NADPH and nicotinamide.

*Oxygen consumption.* This was measured at 37° by using the E.I.L. Clark Bishop oxygen electrode, connected to a Solartron amplifier and Unicam recorder. In most experiments the concentrations of reactants were as described for lipid peroxide studies and the total volume was 1·5–2·0 ml. The rate of $O_2$ uptake was measured for 0·5–2·5 min., and was linear over these short periods. Immediately after completion of the incubation period the reaction mixture was removed for determination of lipid peroxide content.

Unless otherwise specified all values are means of three experiments.

## RESULTS

*Lipid peroxide formation induced by NADPH and ascorbate.* Suspensions of microsomes incubated at pH 6·0 or pH 7·0 form lipid peroxide very slowly during periods of 2 hr. or less, but the rate increases after prolonged incubation even at low temperatures. Thus Wills & Wilkinson (1967) showed that microsomes kept at 4° for 20 hr. produced large quantities of lipid peroxide. The rate of formation of lipid peroxide was unaffected by NADH, but increased greatly by addition of NADPH (40 $\mu$M) or ascorbate (0·5 mM) (Fig. 1). Ascorbate at 10 mM was much less effective than at 1 mM and an optimum concentration was necessary to give a maximum rate of peroxidation. No decrease in the

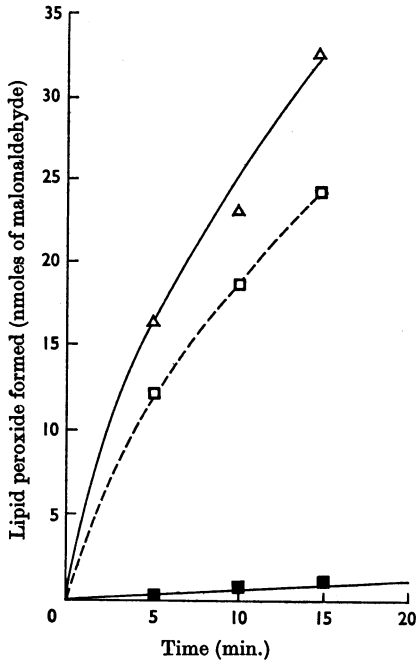


Fig. 1. Effect of NADPH and ascorbate on lipid peroxide formation in microsomes. Suspensions of microsomes (1·5mg. of protein/ml.) were incubated in a medium containing sodium phosphate buffer, pH 7·0 (20mM), and KCl (90mM) at 37° in a total volume of 5·0ml. ■, Control (no addition); □, ascorbate (0·5mM); △, NADPH (40μM).

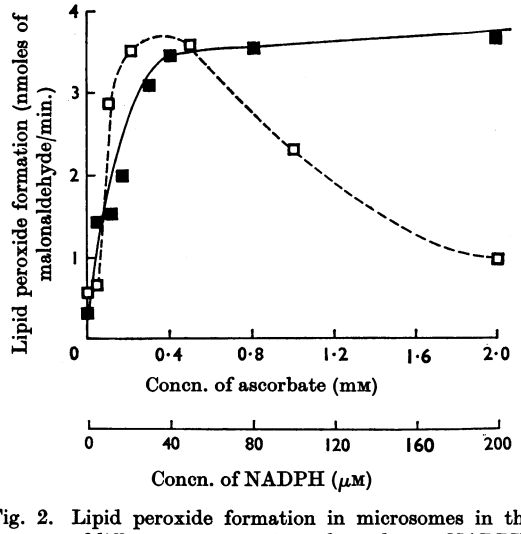


Fig. 2. Lipid peroxide formation in microsomes in the presence of different concentrations of ascorbate or NADPH. Conditions were as described for Fig. 1. □, Ascorbate; ■, NADPH.

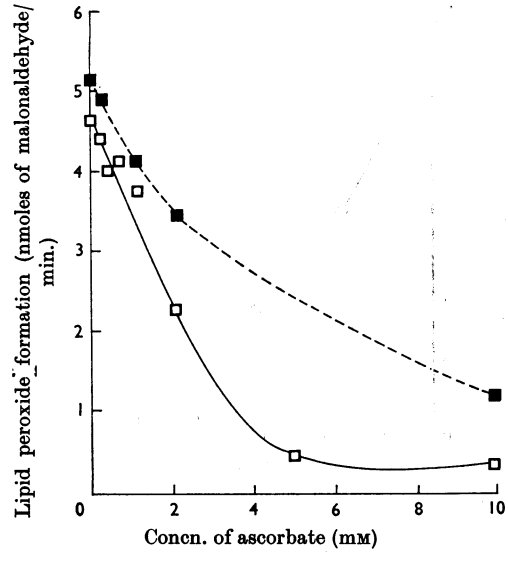


Fig. 3. Effect of ascorbate on lipid peroxide formation in microsomes induced by NADPH. Conditions were as described for Fig. 1. □, 48μM-NADPH; ■, 96μM-NADPH.

rate was observed as the concentration of NADPH was increased (Fig. 2), but this system was inhibited by addition of high concentrations of ascorbate, the extent of the inhibition depending on the NADPH/ascorbate ratio (Fig. 3). In view of these findings 40μM-NADPH or 0·5mM-ascorbate was used in most experiments. Other reducing agents such as cysteine, *p*-phenylenediamine or ferricyanide were not able to replace ascorbate and did not induce lipid peroxide formation when added to microsomes in an equivalent concentration.

*Effect of microsomal concentration on peroxide formation.* Very dilute suspensions of microsomes (1%, 0·15mg. of protein/ml.) form lipid peroxide very rapidly in the presence of NADPH (40μM), but increasing the microsomal concentration beyond 1% did not cause an increase in the specific rate of lipid peroxide formation. Thus the rate of lipid peroxide formation expressed in terms of protein decreased with increase of microsomal protein concentration (Fig. 4).

*Effect of* pH. When microsomes were incubated with ascorbate the optimum pH for lipid peroxide formation was 6·0 and with NADPH the optimum pH was 7·0 (Fig. 5). Determinations over the range pH 6·8–8·0 were also carried out in tris–hydrochloric acid buffer (20mM) and, although the rates, both in the presence of ascorbate and of NADPH, were less than in phosphate buffer, the pH values for an optimum rate were the same as in phosphate buffer.

*Effects of buffers and suspending medium.* Lipid

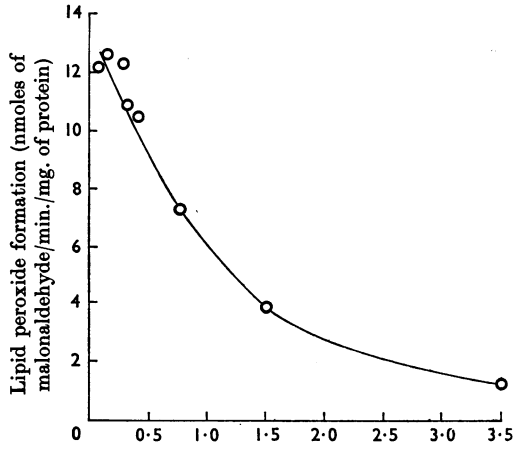


Fig. 4. Effect of microsomal concentration on the rate of peroxide formation. Microsomal suspensions containing between 0·0075 and 3·5 mg. of protein/ml. were incubated at 37° in sodium phosphate buffer, pH 7·0 (20 mM), in the presence of NADPH (40 $\mu$M) and nicotinamide (50 mM).

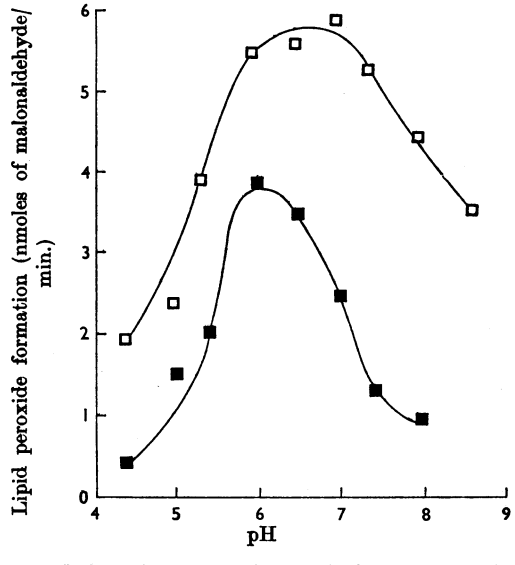


Fig. 5. Effect of pH on lipid peroxide formation in microsomes. Conditions were as described for Fig. 1. The concentration of buffer in each experiment was 25 mM. Sodium acetate buffers were used for the range pH 4·0–5·6, sodium phosphate buffers for pH 5·8–8·0 and tris–HCl buffers for pH 8·0–8·6. □, NADPH system; ■, ascorbate system.

Table 1. *Effect of phosphate on lipid peroxide formation and oxygen uptake by microsomes*

The rate of $O_2$ uptake of a microsomal suspension (1·5 mg. of protein/ml.) in medium containing tris–HCl buffer, pH 7·4 (20 mM), and NADPH (40 $\mu$M) in a total volume of 2·0 ml. was measured in the oxygen electrode for 1–2 min. Sodium or potassium phosphate buffers, pH 7·4, were added to adjust the phosphate concentration. At the end of the incubation the lipid peroxide content of the suspension was measured.

| Addition | Concn. (mM) | Lipid peroxide formation (nmoles of malonaldehyde/min.) | $O_2$ uptake (nmoles/min.) |
|---|---|---|---|
| None | — | 6·20 | 91 |
| Sodium | 10 | 6·50 | 117 |
| phosphate | 20 | 10·22 | 150 |
| buffer, | 50 | 13·20 | 211 |
| pH 7·4 | 90 | 19·70 | 300 |
| Potassium | 10 | 6·03 | 94 |
| phosphate | 20 | 7·30 | 117 |
| buffer, pH 7·4 | 50 | 8·98 | 164 |
| NaCl | 20 | 6·50 | 101 |
| | 90 | 7·70 | 117 |

Table 2. *Effect of suspending media and microsome concentration on lipid peroxide formation and oxygen uptake by microsomes*

After preparation, microsomes were suspended in 0·25 M-sucrose, 0·125 M-KCl, 0·125 M-NaCl or water (1 ml. of medium/g. of liver). Each suspension was then diluted 4:1, 10:1 and 20:1 with the appropriate suspending medium and the rates of $O_2$ uptake and lipid peroxide formation were measured in sodium phosphate buffer, pH 7·0 (final concn. 10 mM), in the presence of NADPH as described in Table 1.

| Suspending medium | Concn. of microsomal preparation (mg. of protein/ml.) | $O_2$ uptake (nmoles/min./mg. of protein) | Lipid peroxide formation (nmoles of malonaldehyde/min./mg. of protein) |
|---|---|---|---|
| Sucrose | 0·92 | 63 | 6·97 |
| (0·25 M) | 1·83 | 55 | 4·48 |
| | 4·58 | 20 | 1·05 |
| KCl | 0·92 | 54 | 7·25 |
| (0·125 M) | 1·83 | 42 | 4·77 |
| | 4·58 | 14 | 1·02 |
| NaCl | 0·92 | 46 | 7·60 |
| (0·125 M) | 1·83 | 27 | 4·45 |
| | 4·58 | 16 | 1·34 |
| Water | 0·92 | 60 | 8·32 |
| | 1·83 | 25 | 4·65 |
| | 4·58 | 19 | 1·29 |

peroxide formation in microsomes incubated in 20 mM-tris buffer, pH 7·4, in the presence of NADPH or ascorbate was strongly stimulated by addition of phosphate buffer, pH 7·4, the buffer containing $Na^+$ being more effective than that containing $K^+$ (Table 1).

In the presence of 10mM-sodium phosphate buffer, pH 7·0, the rate of lipid peroxidation was virtually the same whether the microsomes were suspended in 125mM-sodium chloride or 125mM-potassium chloride, but peroxidation in dilute microsomal suspensions (0·92mg. of protein/ml.) was faster in water and slower in 0·25M-sucrose (Table 2). Fig. 3 shows that the rate of lipid peroxide formation of microsomes suspended in potassium chloride is much greater in dilute suspensions than in concentrated suspensions. The possibility that this effect was associated with the medium, potassium chloride solution, was investigated by studying the rate of lipid peroxide formation at various dilutions in different media. In all media examined increase in the concentration of microsomes was not accompanied by a proportional increase in the rate of lipid peroxide formation or oxygen uptake expressed in terms of microsomal protein (Table 2), and it can therefore be concluded that the rapid rates observed in dilute suspensions result from the actual dilution and are not related to the composition of the suspending medium.

*Relationship between oxygen uptake and peroxide formation.* Lipid peroxide formation in incubated microsomes was always accompanied by oxygen uptake and under a wide range of experimental conditions oxygen uptake increased with increase of formation of lipid peroxide, but a relatively large volume of oxygen was absorbed for each molecule of malonaldehyde produced (Table 3).

*Effects of metal ions.* Inorganic iron ($Fe^{2+}$ or $Fe^{3+}$) increased the rate of peroxide formation, but most other metal ions inhibited when added in low concentrations (Table 4). $Mg^{2+}$ in high concentrations (10mM) increased the rate to a small extent and this was in sharp distinction to $Ca^{2+}$, which inhibited in an equivalent concentration and overcame $Mg^{2+}$ stimulation when both metal ions were present together (Fig. 6).

*Lipid peroxide in boiled microsomes.* Microsomes kept for 1min. at 100°, cooled and then incubated with ascorbate (0·5mM) formed slightly more lipid

Table 3. *Relation between oxygen uptake and lipid peroxide formation in incubated microsomes*

Experimental details were as described for Table 1, but sodium phosphate buffer (20mM) was used in most experiments. Ascorbate (0·5mM) replaced NADPH and nicotinamide in one series and sodium phosphate buffer, pH 6·0, was used in most experiments in which ascorbate was present.

| System | No. of experiments | $O_2$ uptake/malonaldehyde formed molar ratio | |
|---|---|---|---|
| | | Mean | S.D. |
| NADPH | 128 | 15·50 | ±4·60 |
| Ascorbate | 38 | 18·68 | ±7·95 |

Table 4. *Effect of metal ions on lipid peroxide formation by microsomes*

Experimental details were as described for Fig. 1, but the volume of incubated microsomes was 2·0ml. Rates of peroxide formation are expressed as percentages of the control rate.

| Metal ion added | Concn. (mM) | Rate of lipid peroxide formation (%) | |
|---|---|---|---|
| | | Ascorbate system | NADPH system |
| None (control) | — | 100 | 100 |
| $Mg^{2+}$ | 10 | 136 | 140 |
| | 1 | 100 | 100 |
| $Ca^{2+}$ | 10 | 60 | 3 |
| | 1 | 100 | 70 |
| $Zn^{2+}$ | 1 | 69 | 45 |
| | 0·5 | 73 | 65 |
| $Mn^{2+}$ | 1·0 | 4 | 0 |
| | 0·5 | 0 | 0 |
| $Co^{2+}$ | 1·0 | 0 | 2 |
| | 0·5 | 8 | 8 |
| $Cu^{2+}$ | 1·0 | 11 | 13 |
| | 0·5 | 18 | 10 |
| $Hg^{2+}$ | 1·0 | 34 | 1 |
| | 0·5 | 85 | 4 |

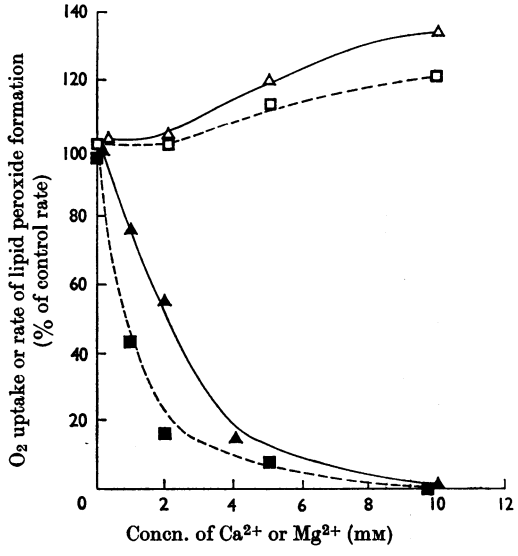


Fig. 6. Effect of $Ca^{2+}$ and $Mg^{2+}$ on $O_2$ uptake and lipid peroxide formation by microsomal suspensions in the presence of NADPH. Experimental details were as described for Table 1 and with tris–HCl buffer (20mM). Results are expressed as percentages of the control rates with no $Ca^{2+}$ or $Mg^{2+}$ added. ▲, $O_2$ uptake in the presence of $Ca^{2+}$; ■, lipid peroxide formation in the presence of $Ca^{2+}$; □, $O_2$ uptake in the presence of $Mg^{2+}$; △, lipid peroxide formation in the presence of $Mg^{2+}$.

Table 5. *Effect of thiol reagents on lipid peroxide formation in normal and boiled microsomes*

'Boiled microsomes' were kept at 100° for 1 min. The period for which the microsomes were in contact with *N*-ethylmaleimide was varied over the range 0–30 min. before starting the experiment. Other experimental details were as described for Fig. 1.

| | | | Lipid peroxide formation (nmoles of malonaldehyde/min.) | |
|---|---|---|---|---|
| | Addition | Concn. (mM) | Ascorbate system | NADPH system |
| Control microsomes | None (control) | — | 3·00 | 5·95 |
| | *p*-Chloromercuribenzoate | 1 | 2·90 | 0 |
| | | 0·5 | 3·14 | 0·73 |
| | Iodoacetamide | 2 | 2·88 | 6·14 |
| | | 1 | 3·15 | 6·56 |
| | *N*-Ethylmaleimide | 2 (0 min.) | — | 4·65 |
| | | (15 min.) | 2·85 | 4·00 |
| | | (30 min.) | — | 3·59 |
| | | 1 (0 min.) | — | 5·62 |
| | | (15 min.) | 3·30 | 5·18 |
| | | (30 min.) | — | 4·20 |
| Boiled microsomes | None (control) | — | 3·57 | 0·03 |
| | *p*-Chloromercuribenzoate | 1 | 1·04 | 0 |
| | | 0·5 | 1·16 | 0 |

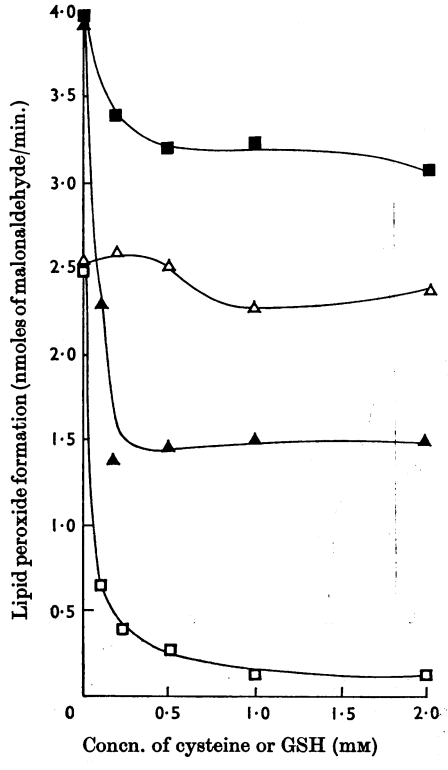


Fig. 7. Effect of GSH and cysteine on lipid peroxide formation in microsomes. Experimental details were as described for Fig. 1. ■, Cysteine added (NADPH system); △, cysteine added (ascorbate system); ▲, GSH added (NADPH system); □, GSH added (ascorbate system).

peroxide than untreated microsomes, but this treatment completely abolished NADPH-stimulated peroxide formation (Table 5).

*Effect of thiol compounds and reagents.* GSH at 0·1 mM inhibited ascorbate-induced peroxidation, but the effect on NADPH-induced peroxidation was much less. Although 0·2 mM-GSH decreased the rate in the presence of NADPH to one-third of the control rate, increasing the GSH concentration to 2 mM did not cause an increase in the inhibition. Cysteine was much less effective. Ascorbate-induced peroxidation was not inhibited to any significant extent, but the addition of 1 mM- or 2 mM-cysteine decreased the rate of NADPH-induced peroxidation by about one-fifth (Fig. 7).

*p*-Chloromercuribenzoate in a low concentration (0·2 mM) strongly inhibited NADPH-induced peroxidation, but had no effect on ascorbate-induced peroxidation. Lipid peroxide formation in boiled microsomes in the presence of ascorbate was, however, strongly inhibited by *p*-chloromercuribenzoate. Other thiol reagents, such as iodoacetamide, did not affect peroxide formation in the presence of NADPH, but *N*-ethylmaleimide inhibited, although to a smaller extent than *p*-chloromercuribenzoate, if the microsomes were in contact with the *N*-ethylmaleimide for at least 15 min. before starting the experiment (Table 5). Inhibition by *p*-chloromercuribenzoate occurred immediately after addition to the microsomes.

*Effect of haematin proteins.* Addition of haemoglobin, cytochrome *c* or haemin in concentrations within the range 0·01–0·1 mM also strongly inhibited lipid peroxide formation and oxygen uptake by

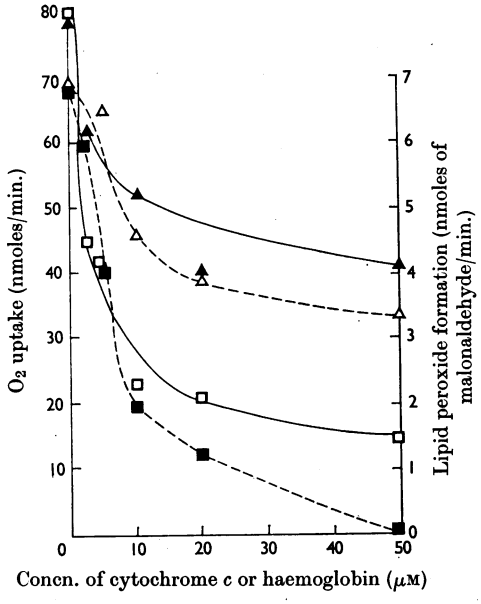


Fig. 8. Effect of haemoglobin and cytochrome *c* on lipid peroxide formation and $O_2$ uptake by microsomes. Suspensions of microsomes were incubated in sodium phosphate buffer, pH 7·0 (20 mM), in the presence of various concentrations of haemoglobin or cytochrome *c*, under the conditions described for Table 1. ▲, $O_2$ uptake in the presence of cytochrome *c*; △, lipid peroxide formation in the presence of cytochrome *c*; □, $O_2$ uptake in the presence of haemoglobin; ■, lipid peroxide formation in the presence of haemoglobin.

Table 6. *Effect of surface-active agents on lipid peroxide formation in microsomes*

Suspensions of microsomes were incubated with ascorbate or NADPH in the presence of various concentrations of surface-active agents. Experimental conditions were as described for Fig. 1.

| Addition | Concn. (mM) | Lipid peroxide formation (nmoles of malonaldehyde/min.) Ascorbate system | NADPH system |
|---|---|---|---|
| None (control) | 0 | 3·36 | 3·52 |
| Deoxycholate | 1·25 | 2·92 | 3·86 |
| | 2·50 | 0·55 | 0·55 |
| | 6·25 | 0·11 | 0·33 |
| | 12·50 | 0 | 0 |
| Sodium dodecyl sulphate | 1 | 0·12 | 1·16 |
| | 2 | 0·44 | 0·99 |
| | 5 | 0·22 | 0·61 |
| Cetylpyridinium bromide | 1 | 0·11 | 0·05 |
| | 2 | 0 | 0 |

microsomal suspensions in the presence of ascorbate or of NADPH, haemoglobin being more effective than cytochrome *c* (Fig. 8).

*Effect of deoxycholate and peroxide formation in microsome fractions.* Dallner (1963) has fractionated microsomes by treatment with 0·275% (w/v) sodium deoxycholate followed by centrifugation for 30 min. at 105000 *g*. In a preliminary study before adoption of this method the effect of deoxycholate in various concentrations was studied on ascorbate- and NADPH-induced lipid peroxide formation and oxygen uptake by microsomal suspensions. In both systems sodium deoxycholate in concentrations greater than 0·1% (w/v) (2·4 mM) strongly inhibited lipid peroxide formation (Table 6). Other detergents such as sodium dodecyl sulphate or cetylpyridinium bromide also inhibited. Dallner's (1963) method was modified by taking 0·1% (w/v) sodium deoxycholate in place of 0·275% (w/v). The rate of peroxide formation in the presence of ascorbate or NADPH was greatest in the fluffy layer, which is believed to consist of endoplasmic reticulum and vesicles (Table 7).

## DISCUSSION

There are several features of the ascorbate and the NADPH systems catalysing lipid peroxidation in microsomes that indicate that a portion at least of each system is common to both. The fact that both processes are associated with the same fraction of the microsomes, the fluffy layer composed of membranes of the endoplasmic reticulum with a high lipid content (Table 7), provides good evidence that the substrates for peroxidation, the unsaturated fatty acids of the membranes, are the same for both systems. Further, destruction of the membrane by detergents abolishes the peroxide formation whether NADPH or ascorbate is used (Table 6). Both systems also require inorganic or non-haem iron in a low concentration (Wills, 1969*a*). Rates of both the ascorbate- and NADPH-induced peroxidation are greater in phosphate buffer than in other buffers (Table 1). The role of phosphate is not certain, but it may associate with iron to form a more active complex (Wills, 1969*a*).

Dilute microsomal suspensions formed much more lipid peroxide/mg. of protein in the presence of ascorbate or NADPH than did concentrated suspensions (Fig. 4), and this may be a result of dispersal of the reticulum in an aqueous phase so that the membranes become extended and the unsaturated lipids lie in close proximity to radicals formed in the aqueous phase. A similar effect was observed in studies of the peroxidation of emulsified tissue lipids exposed to ionizing radiation. Dispersed dilute emulsions formed much more peroxide/mg. of lipid than did concentrated emulsions (Wills & Rotblat, 1964). Alternatively, the increase of the specific rate of peroxidation that accompanies dilution of the microsomal suspension

Table 7. *Lipid peroxide formation in microsomal subfractions*

Microsomal preparations were fractionated into a sediment, fluffy layer and supernatant. The sediment and fluffy layer were resuspended in 0·125 M-KCl (0·5 ml./g. of liver). Rates of lipid peroxide formation were measured as described for Fig. 1.

| System | Fraction | Concn. of protein (mg./ml.) | Lipid peroxide formation: Rate (nmoles of malonaldehyde/min.) | Lipid peroxide formation: Amount (nmoles of malonaldehyde/mg. of protein) |
|---|---|---|---|---|
| NADPH | Whole microsomes | 1·84 | 7·65 | 4·10 |
| | Supernatant | 1·20 | 1·15 | 0·96 |
| | Fluffy layer | 0·89 | 4·30 | 4·82 |
| | Sediment | 0·70 | 0·67 | 0·95 |
| Ascorbate | Whole microsomes | 1·84 | 4·90 | 2·57 |
| | Supernatant | 1·20 | 2·05 | 1·71 |
| | Fluffy layer | 0·89 | 3·96 | 4·45 |
| | Sediment | 0·70 | 0·73 | 1·04 |

may be a result of a decrease in concentration of the haem proteins in the preparation, these being likely to be inhibitory when present in a concentration greater than 0·01 mM (Fig. 8).

Most of the evidence is, however, in favour of the concept that the ascorbate system is much simpler than the NADPH system and may involve non-haem iron as the only other component. Peroxidation of emulsions of pure unsaturated fatty acids takes place readily in the presence of ascorbate and $Fe^{2+}$ (Wills, 1965), and a similar system may be operative in the microsomes. The non-enzymic nature of the process is indicated by the rapid rate of ascorbate-induced peroxidation that occurs in boiled microsomes (Table 5). However, peroxidation in boiled microsomes is, unlike that in untreated microsomes, strongly inhibited by *p*-chloromercuribenzoate, indicating that a new thiol-containing component, possibly a denatured thiol protein, is active as part of the system. Two different processes of peroxide catalysis may therefore be involved in the native and denatured systems when ascorbate is added.

There are, however, many significant points of difference between the two systems. Thus the optimum pH of the NADPH system is close to neutrality, whereas that of the ascorbate system is more acid (Fig. 5). Further, increasing the concentration of ascorbate beyond that necessary for an optimum rate of peroxidation (0·5 mM) decreased the rate, an effect not observed with NADPH (Fig. 2). In addition, high concentrations of ascorbate (10 mM) strongly inhibited lipid peroxide formation in the NADPH system (Fig. 3). If it is assumed that ascorbate functions by keeping a proportion of the iron in a ferrous state and that a balance of the ferrous/ferric iron is essential for maintenance of the autoxidation process (Wills, 1965), then high concentrations of ascorbate could destroy this balance. On the other hand, NADPH transfers its electrons by means of a protein, probably a flavoprotein (Kamin, Masters, Gibson & Williams, 1966), which is able to regulate the electron flow.

Destruction of NADPH-induced peroxidation by boiling the microsomes and the powerful inhibition by *p*-chloromercuribenzoate indicate that the NADPH system is more complex than the ascorbate system and that at least one enzyme, possibly a thiol enzyme, is involved in the process. However, the very strong inhibition of the NADPH system by *p*-chloromercuribenzoate or $Hg^{2+}$ contrasts sharply with the weak effect of *N*-ethylmaleimide and the absence of effect of iodoacetamide on the system (Table 5). These findings indicate that thiol groups of normal reactivity are not present. The inhibition produced by *p*-chloromercuribenzoate or $Hg^{2+}$ may be a result of a combination with the imidazole groups of a protein of the electron-transport chain (cf. Webb, 1966), but it is possible that some sulphur-containing groups of a special type exist in the microsomes and it is highly significant that a similar pattern of reactivity to thiol reagents is found in the bacterial and plant ferredoxins (Malkin & Rabinowitz, 1967). These observations, coupled with those on the role of iron in the system (Wills, 1969*a*), may be reasonably interpreted on the basis that a ferredoxin-type protein forms an essential part of the NADPH system catalysing lipid peroxide formation.

Haem proteins have already been shown to inhibit peroxidation of emulsions of unsaturated fatty acids whether catalysed by metal ions (Lewis & Wills, 1963) or by tissue homogenates (Wills, 1966), and they also inhibit peroxidation of microsomal lipids. Haem proteins are known to be capable of destroying lipid peroxide and so breaking the autoxidation chain (Tappel, 1955), and the inhibi-

tion of all these different systems suggests that a common mechanism for the actual peroxidation of the lipid is operative in all systems. It is not clear why the cytochrome components of the microsome fraction, cytochrome $b_5$ and cytochrome $P$-450, are not inhibitory, but this is possibly on account of their low concentration or spatial relationship to the unsaturated lipids of the reticulum.

The activating effect of inorganic iron (Wills, 1969*a*) contrasts with the effect of most other heavy-metal ions, which inhibited peroxidation induced by ascorbate or by NADPH (Table 4). Inhibition of the NADPH system may be explained to occur as a result of binding the thiol groups or imidazole groups of an essential enzyme, or of NADPH itself or of a ferredoxin-type protein, but inhibition of the ascorbate system cannot be easily explained if this process involves non-protein-bound inorganic iron only. It is possible that these metal ions combine with the phospholipids of the membrane and protect them against oxidation. An alternative explanation is that the form of iron utilized in the ascorbate system is not actually free but loosely bound to protein and either that this protein is denatured by the heavy-metal ions added or that the iron ($Fe^{2+}$ or $Fe^{3+}$) is displaced from its combining sites. The activating effect of $Mg^{2+}$ and the inhibitory effects of $Ca^{2+}$ (Fig. 6) are, however, most likely to be a result of combination with and alteration of the configuration of the membrane phospholipids.

A relatively large quantity of oxygen, between 15 and 19 moles, is absorbed/mole of malonaldehyde produced. In studies of the peroxidation of methyl esters of pure linolenic acid and arachidonic acid, Dahle, Hill & Holman (1962) found a high oxygen uptake/malonaldehyde formed ratio, usually about 12–16:1, and Wills (1966) found a high ratio for peroxidation of emulsions of linoleic acid catalysed by tissue homogenate. Since a proportion of the oxygen will be utilized in the microsomes for the oxidation of linoleic acid and oleic acid and some other unsaturated fatty acids that produce no malonaldehyde (Dahle *et al.* 1962) the oxygen uptake/malonaldehyde formed ratio would be expected to be high. May & McCay (1968) have demonstrated that only a small percentage of the loss of membrane fatty acids can be accounted for as malonaldehyde. Other endogenous non-lipid substrates may also be undergoing oxidation in the incubated microsomes.

The powerful inhibition of ascorbate-induced peroxidation by GSH observed by Christophersen (1968) has been confirmed, but Fig. 7 shows that the effect of thiol compounds on the process is complex and cysteine is virtually without effect on ascorbate-induced peroxidation. GSH decreases the rate of NADPH-stimulated peroxidation, but even in the presence of relatively high concentrations (2mM) peroxidation continues at about one-third the rate of the control. Cysteine on the other hand inhibits NADPH-stimulated peroxidation to a greater extent than it does ascorbate-stimulated peroxidation, but the rate continues at nearly 80% of the control even in the presence of 2mM-cysteine. Thiol compounds can block the peroxidation of emulsions of fatty acids catalysed by haemoglobin (Lewis & Wills, 1963), by reacting with the hydroperoxide and so breaking the autoxidation chain reaction, and it is possible that in the microsomes GSH is oxidized by means of a glutathione peroxidase (Christophersen, 1968). The reason for the great difference between the effects observed when GSH or cysteine acts on ascorbate- and NADPH-induced peroxidation is not clear, for if simple reactions with lipid peroxides were involved the effects should be comparable in the two systems. If dehydroascorbate is an essential intermediate in the ascorbate system GSH may inhibit by reducing this component to ascorbate, a reaction that has been demonstrated in plant tissue by Crook & Morgan (1944). Further, it is possible that the membrane lipids peroxidized by the two systems are not identical and that a portion of the lipids peroxidized by the NADPH system is not available to ascorbate.

Most of the results are, however, in agreement with the concept that the electron-transport chain of the microsomes normally concerned with hydroxylation (Siekevitz, 1966; Ernster & Orrenius, 1966) can be switched to oxidize unsaturated lipids of the endoplasmic reticulum and that this oxidation produces lipid peroxides. Ascorbate supplies electrons at a point along the chain, normally reduced by NADPH, at the site of a non-haem iron complex. The relationship of lipid peroxide formation to hydroxylation is discussed by Wills (1969*b*).

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