Oxidation of Reduced Glutathione by Subcellular Fractions of Rat Liver

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1. A new method was used to diminish the autoxidation of GSH. 2. The oxidation of GSH by liver homogenates was studied with regard to concentration of homogenate, concentration of GSH, time, pH and anaerobiosis. 3. GSH was oxidized by recombinations of the supernatant with microsomes and with mitochondria. Each fraction alone caused little oxidation. 4. Proteins in the supernatant were required to obtain the effect, and low-molecular-weight compounds in the same fraction increased its effect. 5. GSH diminished the formation of malonaldehyde in homogenates. 6. GSH prevented a stimulating effect of the supernatant on the formation of malonaldehyde in microsomes and in mitochondria. 7. The malonaldehyde formation in microsomes together with the supernatant did not start until the concentration of endogenous low-molecular-weight thiols had decreased to a low level. 8. It is suggested that part of the oxidation of GSH in homogenates is coupled to a mechanism that counteracts the peroxidation of membrane lipids.

A series of enzymes catalyse the oxidation of GSH *in vitro*, e.g. glutathione–homocystine transhydrogenase (EC 1.8.4.1) (Racker, 1954), nitroglycerol reductase (Heppel & Hilmoe, 1950), cytochrome *c*–cytochrome oxidase (EC 1.9.3.1) (Keilin & Hartree, 1938; Stotz, Harrer, Schultze & King, 1937–38; Boeri, Baltscheffsky, Bonnichsen & Gustav-Paul, 1953) and catalase (EC 1.11.1.6) (Boeri & Bonnichsen, 1952). It is not known whether these enzymes oxidize GSH *in vivo*.

In erythrocytes glutathione peroxidase (EC 1.11.1.9) catalyses the oxidation of GSH by hydrogen peroxide (Mills & Randall, 1958; Cohen & Hochstein, 1963). This enzyme is also found in the liver and several other organs of the rat (Mills, 1960). It has been suggested that one of the functions of GSH may be the removal of peroxides formed by peroxide-producing enzymes (Mills, 1960; Cohen & Hochstein, 1963). Glutathione peroxidase and contraction factor I of mitochondria (Lehninger & Gotterer, 1960; Lehninger, 1962; Neubert, Rose & Lehninger, 1962) are identical (Neubert, Wojtczak & Lehninger, 1962).

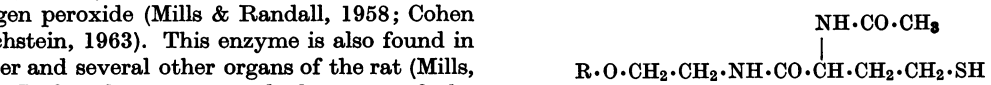
In homogenates of liver and kidney from mice and guinea pigs (Ames & Elvehjem, 1945; Ziegenhagen, Ames & Elvehjem, 1947) and of rat liver (Pinto, 1961) an enzymic oxidation of GSH has been demonstrated. The nature of the reactions has not been established.

The oxidation of GSH by subcellular fractions has previously been little studied.

The autoxidation of GSH in homogenates has been an obstacle to earlier studies (Pinto, 1961). To counteract the autoxidation I have used a new method, which is based on the finding by Jellum (1964) that a cross-linked dextran containing SH groups, synthesized in this Laboratory (Eldjarn & Jellum, 1963), can decrease the autoxidation of GSH when added to incubation media.

METHODS

SH-Sephadex. This was synthesized from Sephadex G-25 (medium grade) (Eldjarn & Jellum, 1963). It contains



side chains anchored to the cross-linked dextran. The thiol content was 170 μmoles of SH group/g., i.e. approx. 1 SH group/35 glucosyl units.

Tissue preparations. Rat liver was homogenized in a Potter–Elvehjem homogenizer at 0° in 4 vol. of 'phosphate-buffered saline' buffer, which consisted of 100 vol. of 0·155 M-KCl, 10 vol. of 0·1 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·4, and 1 vol. of 0·1 M-$MgCl_2$ (Pinto, 1961), and centrifuged for 5 min. at 800*g* at 0°. The supernatant, referred to as 'crude homogenate', contained 33–37 mg. of protein/ml.

A particle-free supernatant containing 18–22 mg. of protein/ml. was prepared from the crude homogenate by centrifuging at 96000*g* for 30 min. in a Spinco model L–50 centrifuge.

The particle-free supernatant was separated into a protein fraction and a fraction containing substances of low molecular weight by gel filtration on a column (35 cm.

× 1·5 cm.) of Sephadex G-25 equilibrated with phosphate-buffered saline and the transmission at 260 mμ of the effluent was recorded with an LKB Uvicord instrument.

Mitochondria were prepared from liver homogenized in 9 vol. of 0·25 M-sucrose. Nuclei and debris were removed by centrifuging at 800*g* for 5 min. and the mitochondria were sedimented by centrifuging the supernatant at 18000*g* for 10 min. The pellet was resuspended and the mitochondria were resedimented by centrifuging at 7000*g* for 10 min.

Microsomes were sedimented from the 18000*g* supernatant by centrifuging at 96000*g* for 30 min.

Particles that were used for the determination of malonaldehyde were washed in 0·155 M-KCl.

Denatured samples were prepared by heating the subcellular fractions in a boiling-water bath for 3 min.

Standard incubation conditions. The subcellular fractions were incubated in phosphate-buffered saline with 100 mg. of SH-Sephadex for 60 min. at 37° in stoppered 15 ml. tubes placed horizontally to allow a good mixing between the medium and the SH-Sephadex particles.

After the incubation the SH-Sephadex was separated from the medium by centrifuging, washed and stored at −20° until examined.

Assay procedures. The protein content of the subcellular fractions was measured by the biuret method.

SH-Sephadex was added to the incubation media for the following reasons.

(1) To diminish the autoxidation of GSH. The effect is due primarily to removal of low-molecular-weight disulphides. The catalytic effect of GSSG on the autoxidation of GSH (Schneider, Smith & Hunter, 1964) is thus prevented. Also, SH-Sephadex forms complexes with heavy metals (Jellum, 1964). The autoxidation of a number of low-molecular-weight thiols such as cysteine and cysteamine can also be diminished in this way (Jellum, 1964).

(2) To keep a constant substrate (GSH) concentration and to avoid accumulation of GSSG.

(3) The amount of GSH oxidized was measured by assay of the disappearance of the SH groups on the SH-Sephadex during the incubation. The method requires the following. (*a*) The GSSG formed must be quantitatively reduced by SH-Sephadex during the incubation. Fig. 1 shows that GSSG was completely reduced by SH-Sephadex and that the reaction rate was considerable at pH 7·5. Fig. 2(*b*) shows that, even with the highest homogenate concentrations used in the present experiments and at the highest rates of oxidation of GSH, the GSH concentration remained constant during incubations at pH 7·4. The rate of the reaction of GSSG with SH-Sephadex can therefore not be rate-limiting in these experiments. In experiments at pH values below 7·0 an initial fall in the GSH concentration was found. SH-Sephadex was added in excess so that 60–70% remained in the reduced state at the end of the incubations. (*b*) It is important that SH-Sephadex should not be oxidized by components other than GSSG during the experiments. Protein disulphide groups did not oxidize SH-Sephadex to any significant extent since, when alone, neither of the subcellular fractions caused oxidation (Table 2). In experiments where endogenous GSH had been removed (Table 3) the addition of GSH was essential to get oxidation of the SH-Sephadex, supporting the view that condition (*b*) is fulfilled.

The thiol content of the SH-Sephadex was measured iodometrically. Since iodine does not oxidize SH-Sephadex directly, tetrathionate, which reacts rapidly, was added and the thiosulphate formed titrated.

The non-protein thiol content of the subcellular fractions, in liver mainly endogenous GSH, was assayed by the method of Ellman (1959) as modified by Jocelyn (1962).

Malonaldehyde was determined by the thiobarbituric acid reaction as described by Ottolenghi (1958). The molar extinction coefficient ϵ_{535} $1{\cdot}56 \times 10^5$ was used (Sinnhuber, Yu & Yu, 1958).

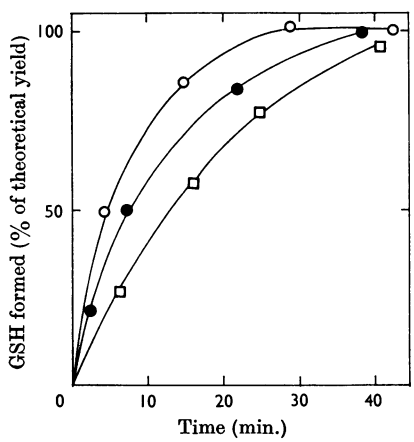


Fig. 1. Reduction of GSSG by SH-Sephadex. A 100 mg. portion of SH-Sephadex was incubated in 4 ml. of 0·05 M-phosphate buffer at 37°. The reaction was started by adding 2·5 μmoles of GSSG. At intervals the medium was quickly centrifuged and 0·2 ml. of the supernatant was removed and frozen in methanol–solid CO_2 until the GSH was determined iodometrically. The pH of the incubation medium was: □, pH 7·0; ●, pH 7·5; ○, pH 8·0.

RESULTS

Fig. 2(*a*) shows that GSH is oxidized by crude rat-liver homogenate, and that the oxidation rate increases nearly linearly with increasing concentrations of the homogenate. The non-enzymic oxidation of GSH by denatured samples of the crude homogenate was approx. 20% of that observed with untreated samples. This low rate is probably due to the use of thiolated Sephadex since other authors have reported a rate two to three times as high (Pinto, 1961).

Fig. 2(*b*) demonstrates that GSH is stable in the media. After a lag period of approx. 10 min. the amount of GSH oxidized increased nearly linearly with time for more than an hour. Under anaerobic conditions the oxidation of GSH was zero.

Even without added GSH a substantial oxidation was found (Fig. 2*c*). This is probably due to the endogenous GSH of rat liver, which corresponds to a concentration of 0·4–0·9 mM (Woodward, 1935;

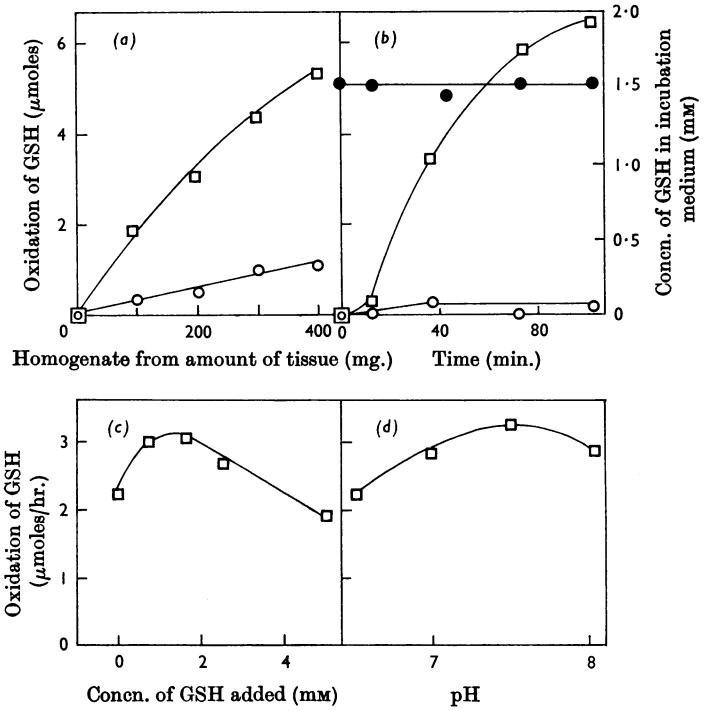


Fig. 2. Oxidation of GSH by the crude homogenate. The concentration of added GSH was 1·2mM, the incubation volume was 2ml. and the standard incubation conditions were used unless stated otherwise. (*a*) Effect of increasing homogenate concentrations. Crude homogenate from 100mg. of liver contained 17mg. of protein. □, Untreated homogenate; ○, boiled homogenate. (*b*) GSH concentration in the media and the oxidation of GSH as a function of time, and effect of anaerobiosis. Crude homogenate (56mg. of protein) from 300mg. of liver was used. ●, Concentration of added GSH. The incubation volume was 4ml. After centrifugation of the media 2ml. of supernatant was removed and the concentration of added GSH determined by the difference in the iodine consumption of media to which GSH (1·5mM) had been added and media with no exogenous GSH. □, Oxidation of GSH. ○, Oxidation of GSH under anaerobic conditions obtained by bubbling N_2 through the media. (*c*) Effect of concentration of added GSH on enzymic oxidation. Crude homogenate (33mg. of protein) from 200mg. of liver was used. (*d*) Effect of pH on enzymic oxidation. Crude homogenate (36mg. of protein) from 200mg. of liver was incubated in 0·1M-KH_2PO_4–Na_2HPO_4 buffers in a volume of 3ml.

Bhattacharya, Robson & Stewart, 1956; Grunert & Phillips, 1951). The highest oxidation rate was found when the concentration of added GSH was 1·2mM, with inhibition at higher concentrations.

The pH optimum of the enzymic oxidation of GSH was broad (Fig. 2*d*). The values obtained below pH7 should be considered minimal since the GSH concentration decreased during incubation in such media. The reduction by the SH-Sephadex of the GSSG formed thus appeared to be incomplete.

Glutathione occurs predominantly in the reduced form in the intact liver (Bhattacharya, Robson & Stewart, 1955). Table 1 shows that NADP (the cofactor of glutathione reductase) and nicotinamide, which is known to inhibit the nucleotidases splitting NADP (Purvis, 1960), markedly diminished the observed oxidation of GSH. It thus seems clear that the enzymic reduction of GSSG is considerably diminished in the homogenate. Isocitrate added to regenerate reduced NADP did not decrease the oxidation of GSH more than NADP

Table 1. *Effect of nicotinamide and NADP on the oxidation of GSH by crude homogenate*

Crude homogenate (33mg. of protein) from 200mg. of liver was used. GSH was added at a concentration of 1·2mM, the incubation volume was 2ml. and the standard conditions were used.

Addition to incubation medium	Oxidation of GSH (μmoles/hr.)
None	3·6
Nicotinamide (50mM)	1·8
NADP (0·5mM)	1·3

Table 2. *Oxidation of GSH by subcellular fractions and by recombinations of these fractions*

All homogenate fractions were added in amounts corresponding to 300 mg. of liver. GSH was added at a concentration of 1·2 mM, the incubation volume was 2 ml. and the standard conditions were used. The protein content of the fractions was: in the crude homogenates 51–56 mg., the particle-free supernatants 30–33 mg., the microsomes 10–11 mg. and the mitochondria 10–11 mg.

Subcellular fraction	Oxidation of GSH (μmoles/hr.) Expt. I	Expt. II	Expt. III
Crude homogenate	4·6	5·7	5·5
Supernatant	0·3	0	0·2
Microsomes	0·1	0	0
Mitochondria	0	−0·2	0
Supernatant + microsomes	3·7	4·8	3·8
Supernatant + mitochondria	2·1	3·4	2·5
Microsomes + mitochondria	0·1	0	0·2
Boiled supernatant + microsomes	0·5	0·1	0·2

Table 3. *Oxidation of GSH by fractions of the particle-free supernatant obtained by gel filtration together with microsomes*

The portions of the particle-free supernatant (35 mg. of protein), the protein fraction of the gel filtrate of the supernatant (33 mg. of protein) and the microsomes (9 mg. of protein) all corresponded to 300 mg. of liver; 2 ml. portions of the low-molecular-weight fraction of the gel filtrate of the supernatant (no detectable protein) were used. GSH was added at a concentration of 1·2 mM, the incubation volume was 4 ml. and the standard conditions were used.

Subcellular fraction	Oxidation of GSH (μmoles/hr.)
Supernatant + microsomes	3·2
Protein fraction of gel filtrate + microsomes	1·3
Low-molecular-weight fraction of gel filtrate + microsomes	0·4
Protein fraction of gel filtrate + low-molecular-weight fraction of gel filtrate	0·2
Protein fraction of gel filtrate + low-molecular-weight fraction of gel filtrate + microsomes	2·4

alone. Probably the homogenate contains sufficient endogenous substrates for the reduction.

The oxidation of GSH by mitochondria, microsomes, particle-free supernatant and recombinations of the fractions is shown in Table 2. Each fraction alone had little ability to oxidize GSH, but recombinations of the microsomal fraction or the mitochondrial fraction with the supernatant showed a high activity.

The finding that boiled particle-free supernatant combined with microsomes or mitochondria oxidized little GSH suggests that its effect is mainly enzymic. This view is supported by the finding that the protein fraction of the gel filtrate of the supernatant caused a significant oxidation of GSH when combined with microsomes (Table 3). The low-molecular-weight fraction of the gel filtrate caused only an insignificant oxidation (Table 3) when incubated together with microsomes, but it increased the oxidation in media that contained both microsomes and the protein fraction, suggesting that it may contain cofactors or substrates of significance for maximal effect.

Since the endogenous GSH had been removed from the protein fraction by the gel filtration and from the microsomes during isolation, it was necessary to add GSH to obtain oxidation of the SH-Sephadex in media that contained these two fractions only.

It has been proposed (Jocelyn, 1964) that a substantial part of the oxidation of GSH in rat-liver homogenate is linked to the oxidation of hypoxanthine and similar substrates by xanthine oxidase.

This possibility is not excluded by the finding that exogenous hypoxanthine decreased the oxidation (Fig. 3), since the homogenate may contain endogenous substrates in optimum concentration for this enzyme.

Xanthine oxidase is located in the particle-free supernatant (Schein & Young, 1952; Villela, Mitidieri & Affonso, 1955). Fig. 3 shows that GSH was rapidly oxidized when hypoxanthine was added to the protein fraction of the particle-free supernatant. The total particle-free supernatant would be expected to contain any endogenous hypoxanthine and similar substrates in addition to the necessary enzymes to couple the oxidation of hypoxanthine to the oxidation of GSH, but caused little oxidation of GSH (Table 2). From these findings it is clear that the oxidation of GSH in the crude homogenate and in the media with

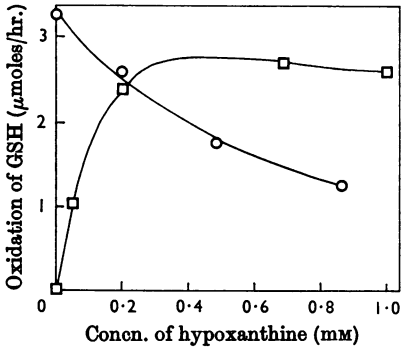


Fig. 3. Effect of hypoxanthine on the oxidation of GSH. GSH was added at a concentration of 1·2 mM, the incubation volume was 4 ml. and the standard conditions were used. ○, Crude homogenate (36 mg. of protein) from 200 mg. of liver; □, protein fraction of the gel filtrate of the particle-free supernatant (24 mg. of protein).

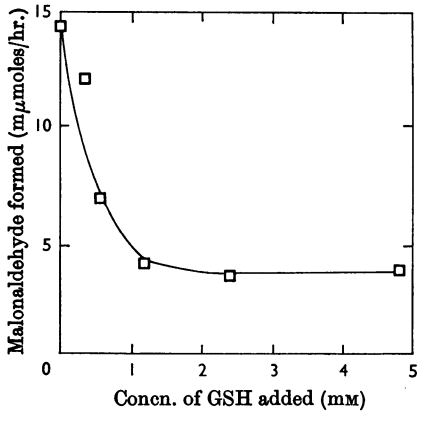


Fig. 4. Effect of GSH on the formation of malonaldehyde by crude homogenate. Homogenate (16 mg. of protein) from 100 mg. of liver was incubated in 2 ml. of phosphate-buffered saline at 37° for 1 hr.

microsomes or mitochondria combined with particle-free supernatant cannot be caused by the xanthine-oxidase system.

Since both microsomes and mitochondria are membrane structures, rich in unsaturated fatty acids, a relation between peroxidation of such fatty acids and the oxidation of GSH appeared possible. Fig. 4 shows that GSH added to the crude homogenate decreased the formation of malonaldehyde, which is known to be formed by peroxidation of polyunsaturated fatty acids (Bernheim, Bernheim & Wilbur, 1948; Sinnhuber *et al.* 1958). The concentration of crude homogenate was critical, with

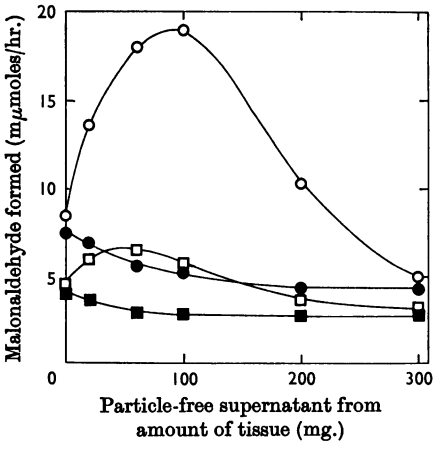


Fig. 5. Effect of GSH on the formation of malonaldehyde in subcellular particles incubated with increasing concentrations of particle-free supernatant. Microsomes (13 mg. of protein) and mitochondria (11 mg. of protein) from 300 mg. of liver were used. Particle-free supernatant from 100 mg. of liver contained 9 mg. of protein. GSH was added at a concentration of 1·2 mM when stated. The fractions were incubated in 2 ml. of phosphate-buffered saline at 37° for 1 hr. ○, Microsomes; ●, microsomes with GSH; □, mitochondria; ■, mitochondria with GSH.

inhibition of the peroxidation with more than approx. 12 mg. of protein/ml.

Fig. 5 shows that the particle-free supernatant stimulated the peroxidation of fatty acids of both microsomes and mitochondria, and that GSH prevented this stimulation. The inhibition of the peroxidation at high concentrations of particle-free supernatant may be caused by endogenous GSH. After isolation both microsomes and mitochondria contained some malonaldehyde. When particle-free supernatant was not present, this amount did not increase significantly during incubation. Boiled particle-free supernatant did not stimulate the peroxidation.

Fig. 6 shows that the addition of GSH after 30 and 45 min. of preincubation stopped the peroxidation. The peroxidation of microsomes together with particle-free supernatant did not start until the concentration of endogenous low-molecular-weight thiol had fallen to a low and relatively constant level. Since GSH is the main low-molecular-weight thiol of liver, the disappearance of low-molecular-weight thiol presumably represents GSH oxidation. It was found that the lag period before the peroxidation started increased with increasing amounts of particle-free supernatant. The exhaustion of the supply of endogenous low-molecular-weight thiol in all experiments closely correlated with the start of peroxidation.

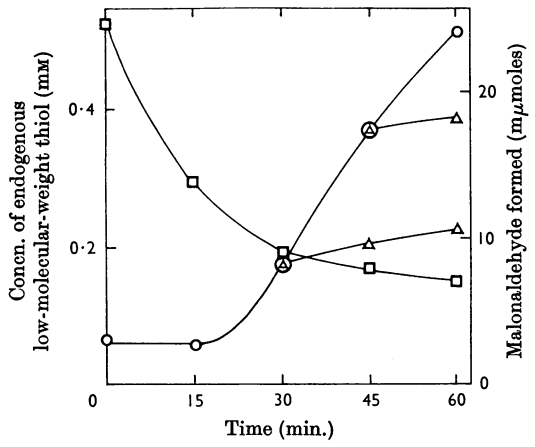


Fig. 6. Concentration of endogenous low-molecular-weight thiol correlated with malonaldehyde formation. Effect of adding GSH after long preincubation periods. Microsomes (12mg. of protein) from 300mg. of liver were incubated together with particle-free supernatant (10mg. of protein) from 100mg. of liver in 2ml. of phosphate-buffered saline at 37°. □, Concentration of endogenous low-molecular-weight thiol; ○, malonaldehyde formation; △, malonaldehyde formation in media with GSH (1·2mM) added after 30 and 45min. of incubation.

DISCUSSION

Although glutathione occurs in high concentrations in animal tissues *in vivo* and the enzymic reduction of GSSG is a well-characterized reaction, little is known about the pathway of GSH oxidation.

GSH prevented the formation of malonaldehyde from the polyunsaturated fatty acids of mitochondria and microsomes in the same systems that caused oxidation of GSH (crude homogenate or particle-free supernatant together with microsomes or with mitochondria). Singly, microsomes, mitochondria and supernatant oxidized little GSH and there was little prevention of malonaldehyde formation. GSH does not affect the thiobarbituric acid reaction with malonaldehyde (Wilbur, Bernheim & Shapiro, 1949). The findings support the view that a substantial part of the oxidation of GSH in liver homogenate is coupled to a mechanism that counteracts peroxidation of membrane lipids.

The effect of GSH can hardly be explained as a removal of preformed factors in the particle-free supernatant that may cause peroxidation, since the formation of malonaldehyde started as soon as the supply of GSH was exhausted. It may be noted that Hunter *et al.* (1964), in experiments with mitochondria, have shown that hydrogen peroxide does not induce lipid peroxidation.

If factors that cause peroxidation are continuously produced during incubation, the effect of GSH may be due to inactivation of such factors.

A second possibility is that GSH reacts either spontaneously or enzymically with some organic peroxide intermediate in the chain of reactions that form malonaldehyde from polyunsaturated fatty acids. This explanation agrees with the tendency of GSH to react with peroxides.

Such a mechanism may protect membranes and other cell constituents *in vivo* against the highly reactive lipid peroxides.

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