Mechanisms of Lipid Peroxide Formation in Animal Tissues

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1. Homogenates of rat liver, spleen, heart and kidney form lipid peroxides when incubated *in vitro* and actively catalyse peroxide formation in emulsions of linoleic acid or linolenic acid. 2. In liver, catalytic activity is distributed throughout the nuclear, mitochondrial and microsomal fractions and is present in the 100000*g* supernatant. Activity is weak in the nuclear fraction. 3. Dilute (0·5%, w/v) homogenates catalyse peroxidation over the range pH 5·0–8·0 but concentrated (5%, w/v) homogenates inhibit peroxidation and destroy peroxide if the solution is more alkaline than pH 7·0. 4. Ascorbic acid increases the rate of peroxidation of unsaturated fatty acids catalysed by whole homogenates of liver, heart, kidney and spleen at pH 6·0 but not at pH 7·4. 5. Catalysis of peroxidation of unsaturated fatty acids by the mitochondrial and microsomal fractions of liver is inhibited by ascorbic acid at pH 7·4 but the activity of the supernatant fraction is enhanced. 6. Inorganic iron or ferritin are active catalysts in the presence of ascorbic acid. 7. Lipid peroxide formation in linoleic acid or linolenic acid emulsions catalysed by tissue homogenates is partially inhibited by EDTA but stimulated by *o*-phenanthroline. 8. Cysteine or glutathione (1mM) inhibits peroxide formation catalysed by whole homogenates, mitochondria or haemoprotein. Inhibition increases with increase of pH.

Linoleic acid peroxide and peroxides of other unsaturated fatty acids are extremely toxic when injected into mice (Horgan, Philpot, Porter & Roodyn, 1957). This toxicity may be a result of oxidative destruction of thiol groups of amino acids and proteins (Lewis & Wills, 1962) and particularly of enzymes (Wills, 1961). Lipid peroxide formation may also result in membrane damage, as has been shown for erythrocytes (Tsen & Collier, 1960) and lysosomes (Wills & Wilkinson, 1966). Formation of lipid peroxides *in vivo* would therefore be very likely to cause severe cellular damage. Under certain conditions, lipid peroxides may be formed *in vivo* but accurate analysis is difficult and reported results are controversial (Zalkin & Tappel, 1960; Bieri & Anderson, 1960; Philpot, 1963). Nevertheless it is firmly established that homogenates of most animal tissues form thiobarbituric acid reactants (commonly called 'peroxides') when incubated *in vitro* under aerobic conditions (Zalkin & Tappel, 1960). These substances are believed to be formed by the oxidation of unsaturated fatty acids of lipid constituents of the tissues probably via hydroperoxides (Holman, 1954). Such studies *in vitro* are clearly useful for the formulation of possible mechanisms of peroxide formation *in vivo*.

Two separate systems have been implicated in the catalysis of lipid peroxide formation in tissues: haemoproteins such as haemoglobin or cytochrome *c* are well established as active catalysts *in vitro* (Tappel, 1955), but iron in the non-haem form has frequently been considered to be more important as a catalyst in tissues (Bernheim, 1963). Peroxide formation in tissues is stimulated by ascorbic acid (Wolfson, Wilbur & Bernheim, 1956) and is inhibited by EDTA (Barber, 1963).

The value of studies on lipid peroxide formation in incubated tissue homogenates is limited by two main considerations. First, lipid peroxide formation in tissues clearly requires substrates, the unsaturated fatty acids of the tissue lipids, and a catalytic system. Either of these factors may limit or control the extent of peroxide formation. Secondly, nearly all investigations have, so far, relied on the thiobarbituric acid colorimetric method for peroxide determination. This method does not measure lipid peroxide directly but actually measures the concentration of malonaldehyde or other degradation products such as 2,4-dienals or 2-enals, which are split from peroxidized unsaturated fats. During the initial stages of peroxidation of pure unsaturated fatty acids such as linolenic acid this 'thiobarbituric acid value' is directly proportional to the peroxide value (Wills & Rotblat, 1964) but the thiobarbituric acid method may be unreliable under certain conditions (Philpot, 1963; Wills, 1964*a*).

To overcome some of these difficulties the

catalytic systems of tissues have been studied by the addition of excess of substrate in the form of emulsions of unsaturated fatty acids to tissue homogenates. This procedure enables other methods of studying oxidation and peroxide formation, such as the measurement of oxygen uptake, to be used and to be compared with the thiobarbituric acid method. Attempts have been made to assess the relative importance of haemoprotein catalysis and inorganic (non-haem) iron catalysis in various tissues by comparing results with those found for these catalysts in model systems (Wills, 1965). Rates of peroxide formation catalysed by various tissues have been compared and effects of varying the experimental conditions and of adding chelating agents examined. A preliminary account of this work has been published (Wills, 1964*b*).

EXPERIMENTAL

Linoleic acid and linolenic acid emulsions. These were prepared as previously described (Wills, 1965).

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

Ferritin. This was prepared from pig spleen as described by Michaelis (1947). Crystalline haemoglobin was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and cytochrome *c* from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Tissues homogenates. Fresh rat tissues were homogenized in 0·25 M-sucrose immediately after the death of the animal. A tissue concentration of 10% or 20% (w/v) was normally prepared.

Oxidation studies. The rates of peroxidation of emulsions of linoleic acid or linolenic acid were studied manometrically as described previously (Wills, 1965). In a typical experiment 2·0 ml. of fatty acid emulsion (0·02 M) in phosphate or tris–HCl buffer was mixed with 0·3 ml. of tissue homogenate (10%, w/v) and the rate of O_2 uptake determined at 37° for 60 min. in a total volume of 3 ml. When linolenic acid oxidation was studied samples (0·2 ml.) were taken immediately after mixing homogenates and emulsion and then again after 60 min. incubation for determination of the lipid 'peroxide' concentration by the thiobarbituric acid method (Wilbur, Bernheim & Shapiro, 1949). This colorimetric method is not suitable for the study of linoleic acid peroxidation (Wilbur *et al.* 1949).

The original method of Wilbur *et al.* (1949) utilized 2·0 ml. of sample with 1·0 ml. of 20% (w/v) trichloroacetic acid and 2·0 ml. of 0·67% (w/v) thiobarbituric acid. In the present investigation it was usually desirable to decrease the sample size to 0·2 ml. or 0·5 ml. and make the reaction volume up to 5·0 ml. Although it is possible, when studying peroxidation of pure linolenic acid, to convert E_{535} values into molar concentrations of peroxide (Wills & Rotblat, 1964), it is likely that such a conversion is not always justified when lipid peroxide concentrations in tissue homogenates are measured by this method. Values obtained by this method have therefore been recorded as the $E^{1cm.}_{535}$ values of a 1 ml. sample, and are described as thiobarbituric acid values. In a few experiments peroxide concentrations were determined by the ferric thiocyanate method (Wagner, Clever & Peters, 1947) or by measuring E_{232} (Holman, 1954). For the latter measurements the emulsions were clarified by diluting with a suitable volume of 0·01 N-NaOH in 10% (v/v) ethanol.

Iron content. Iron contents of tissues were determined on tissue homogenates by the method described by Ramsay (1951, 1954). Haem iron was obtained by difference.

Liver fractions. Fresh rat liver (8 g.) was homogenized and fractionated by the method of Aldridge (1957). Each tissue fraction was resuspended in 8 ml. of 0·25 M-sucrose and the rates of peroxidation of linoleic acid emulsion and linolenic acid emulsion were compared with that of the original homogenate. Nuclei were purified by centrifuging at 50000*g* for 1 hr. as described by Widnell & Tata (1964).

Cytochrome c. Cytochrome *c* concentration in mitochondria was decreased by the method of Jacobs & Sanadi (1960).

RESULTS

Relationship between lipid peroxide values obtained by different methods

The thiobarbituric acid colorimetric method is considered by some workers to be unreliable, or even to determine substances unrelated to lipid peroxide (Philpot, 1963). It was therefore considered desirable to compare measurements of lipid peroxide formation by several different analytical methods. The rate of peroxidation of linolenic acid emulsion catalysed by haemoglobin and tissue homogenates was studied by measuring the oxygen uptake, the peroxide value by the ferric thiocyanate method and E_{232}, and comparing these values with those obtained by the thiobarbituric acid method. The results (Table 1) show that increases in all four quantities are approximately proportional whether the fatty acid peroxidation is catalysed by liver or heart homogenate or by haemoglobin.

Catalysis of peroxidation by different tissues

Effect of homogenate concentration. At pH 6·0 in phosphate buffer homogenates of rat heart, spleen, liver or kidney catalysed a rapid rate of peroxidation of linoleic acid or linolenic acid emulsion. The rate of peroxidation increased with homogenate concentration when dilute tissue suspensions were used but suspensions greater than 4% (w/v) were less effective than 2% (w/v) suspensions. Highly concentrated suspensions of spleen were most inhibitory (Fig. 1). Increase in oxygen uptake was normally paralleled by increase in the thiobarbituric acid value for all four tissues studied (Table 2) and about 60 μl. of oxygen was necessary to increase the thiobarbituric acid value by 1 unit. This absorption of 60 μl. of oxygen corresponds to an uptake of 0·89 μmole of oxygen/ml. It has been calculated (Wills & Rotblat, 1964) that a thio-

Table 1. *Relationship between peroxide formation in linolenic acid emulsion determined by several methods*

Linolenic acid emulsion (16·7mM) in 0·02M-phosphate buffer, pH6·4, was incubated at 37° with liver or heart homogenate (final concn. 0·5%, w/v) or with haemoglobin (final concn. 5μM) in a total volume of 3·0ml. for 60min. Samples (0·2ml.) were taken for determination of peroxide value by the ferric thiocyanate method, thiobarbituric acid (TBA) method and for measurement of E_{232} at the start and end of the incubations. Rates of increase of each measurement observed during the incubation are recorded.

Tissue homogenate	O_2 uptake (μmole/ml./min.)	Increase of peroxide value by ferric thiocyanate method (μmole of hydroperoxide/ml./min.)	Increase of TBA value (E_{535}/ml./min.)	Increase of E_{232} (E_{232}/min.)
Haemoglobin	0·063	0·065	0·080	0·368
Liver	0·038	0·045	0·048	0·254
Heart	0·040	0·041	0·043	0·246

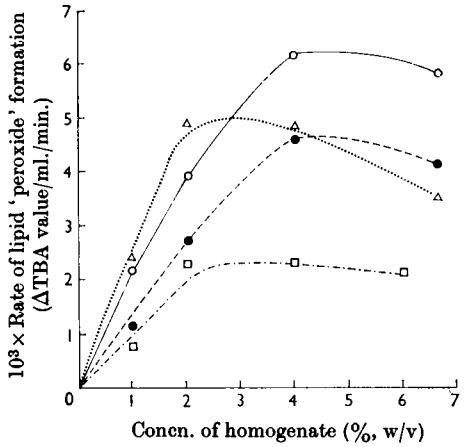

Fig. 1. Relation between homogenate concentration and the rate of lipid 'peroxide' formation in linolenic acid emulsion. Linolenic acid emulsion (16·7mM) in 0·02M-phosphate buffer, pH6·0, was incubated at 37° for 60min. with the tissue homogenate in a total volume of 3ml. Samples (0·2ml.) were removed for the determinations of the thiobarbituric acid (TBA) value (as E_{535}) immediately after adding the homogenate and then again after 60min. incubation. ○, Heart; △, spleen; ●, liver; □, kidney.

Table 2. *Rates of peroxidation of linolenic acid emulsion catalysed by tissue homogenates measured by oxygen uptake and by the thiobarbituric acid method*

Linolenic acid emulsion (16·7mM) in 0·02M-phosphate buffer, pH6·0, was incubated at 37° for 60min. with the tissue homogenate (final concn. 1%, w/v) in a total volume of 3ml. Samples (0·2ml.) were removed for determination of the thiobarbituric acid value immediately after adding the homogenate and again after 60min. incubation. Thiobarbituric acid (TBA) values are calculated for total volume (3ml.). O_2 uptake was followed manometrically.

Tissue homogenate	Rate of peroxidation: Increase of TBA value (E_{535}/min.)	Rate of peroxidation: O_2 uptake (μl./min.)	$\frac{O_2 \text{ uptake}}{\Delta\text{TBA value}}$
Heart	0·0405	2·53	62·5
Spleen	0·0688	4·06	59·0
Kidney	0·0465	2·58	55·6
Liver	0·0423	3·02	71·5

barbituric acid value of 1 corresponds closely to a peroxide concentration of 1·4μmoles/ml. in oxidized linolenic acid when peroxide concentration is determined by measuring E_{232}. The discrepancy between the two values was more marked when ascorbic acid was present.

Effect of pH. At pH6·0, the rate of peroxidation of fatty acid emulsions catalysed by liver homogenate increased with homogenate concentration until the final homogenate concentration was 4% (w/v). At pH7·4, however, the rate of peroxidation catalysed by liver homogenate in a concentration greater than 1% (w/v) was always less than the rate catalysed by a 1% (w/v) homogenate (Fig. 2). With dilute homogenates (0·5%, w/v) the rate of peroxidation of linoleic acid or linolenic acid was little affected over the range pH5·5–8·0. If, however, the concentration of homogenate was high (5%, w/v) the pH of the reaction medium exerted a pronounced effect on the rate of peroxidation. This rate was very rapid in acid solutions (pH5·8) but fell sharply as the solution was made more alkaline, so that at pH8·0 there was very little catalytic activity. At pH8·0 the catalytic activity

of the dilute (0·5%, w/v) kidney homogenate was at least six times that of the concentrated (5%, w/v) homogenate (Fig. 3). Essentially similar results were obtained with homogenates of heart or spleen.

Effect of ascorbic acid. Tissue homogenates incubated in the presence of ascorbic acid form much more lipid 'peroxide', as measured by the thiobarbituric acid method, than when no ascorbic acid is added (Wolfson *et al.* 1956). Further, tissues from ascorbic acid-deficient guinea pigs form less 'peroxide' after incubation than normal tissues do (Abramson, 1949). Ascorbic acid had little effect on the rate of peroxidation of linolenic acid catalysed by spleen homogenates, but when added to liver, kidney or heart homogenate (1%, w/v) in phosphate buffer, pH 6·0, it caused marked increases in the rate of peroxidation of the linolenic acid and in the thiobarbituric acid values (Table 3). Although ascorbic acid increased the rate of peroxidation of unsaturated fatty acids in solutions more acid than pH 6·0 it inhibited the initial rate

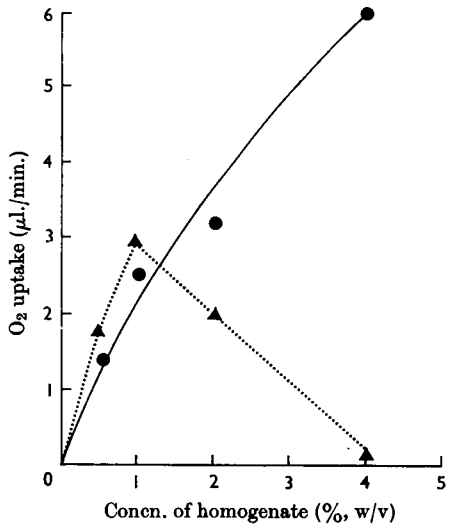

Fig. 2. Relation between concentration of liver homogenate and the rate of linoleic acid peroxidation at pH 6·0 and pH 7·4. Linoleic acid emulsion (16·7 mM) in 0·02 M-phosphate buffer, pH 6·0 or 7·4, was incubated at 37° for 60 min. with liver homogenate in a total volume of 3 ml. O_2 uptake was followed manometrically. ●, pH 6·0; ▲, pH 7·4.

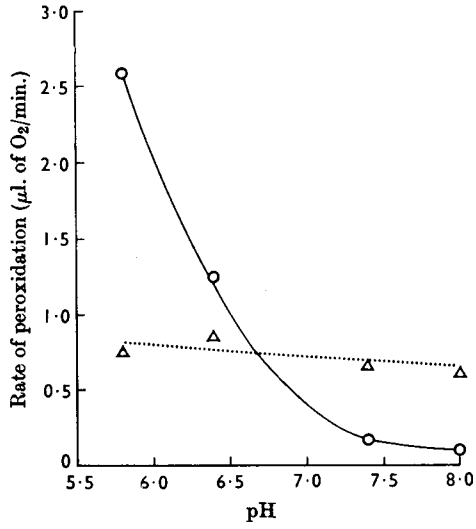

Fig. 3. Effect of pH on the rate of peroxidation of linoleic acid catalysed by kidney homogenate. Experimental details were as described in Fig. 2. ○, 5% (w/v) kidney homogenate; △, 0·5% (w/v) kidney homogenate.

Table 3. *Effect of ascorbic acid on the rate of linolenic acid peroxidation catalysed by tissue homogenates*

Experimental details were as described in Table 2.

		TBA value (E_{535})		O_2 uptake	
Tissue homogenate	Ascorbic acid (1 mM)	Increase/min.	Increase due to ascorbic acid	μl./min.	Increase due to ascorbic acid
Heart	–	0·0405		2·53	
	+	0·133	3·28×	4·47	1·76×
Spleen	–	0·0688		4·06	
	+	0·0742	1·07×	4·85	1·19×
Kidney	–	0·0465		2·58	
	+	0·132	2·84×	4·51	1·75×
Liver	–	0·0423		3·02	
	+	0·0922	2·19×	3·97	1·31×

of peroxidation if the reaction was made more alkaline. For example, the initial rate of peroxidation of linoleic acid catalysed by liver homogenate (1%, w/v) was 85% inhibited by ascorbic acid (1mM) in phosphate or tris–hydrochloric acid buffer, pH 7·4, but increased 1·35-fold at pH 6·0 or pH 5·4.

Effect of destruction of haemoproteins. By treating tissues with warm hydrogen peroxide (Ramsay, 1951) haemoproteins are destroyed. Any catalytic function of haemoproteins should therefore be completely destroyed by prior treatment with hydrogen peroxide. The liberated inorganic iron is a very weak catalyst as compared with an equivalent quantity of haem iron (Wills, 1965).

Treatment of homogenates of heart, spleen, liver and kidney with hydrogen peroxide for 5min. at 50° decreased the catalytic activity for peroxidation of linoleic acid to a small percentage of the original activity. Addition of ascorbic acid to the treated homogenates caused a rapid rate of peroxidation (Table 4) owing to the formation of an active catalytic system with the non-haem iron.

Effect of chelating agents. The rate of iron-catalysed peroxidation of linoleic acid emulsions is strongly stimulated by *o*-phenanthroline but strongly inhibited by EDTA; the haemoprotein-catalysed oxidation is, on the other hand, unaffected by chelating agents (Wills, 1965). Addition of chelating agents to tissue homogenates should therefore help to elucidate the nature of the catalytic system in the tissue.

EDTA (0·2mM) strongly inhibited linolenic acid peroxidation catalysed by kidney and liver homogenates and weakly inhibited the peroxidation catalysed by heart and spleen homogenates (Table 5). Increases in the rate of 'peroxide' formation caused by the addition of ascorbic acid were, however, almost completely abolished by EDTA (Table 5). Therefore a heavy metal, which is likely to be non-haem iron, must play a vital role in such reactions.

Endogenous lipid 'peroxide' formation, measured by the thiobarbituric acid method, in tissue homogenates (1%) incubated in the presence of ascorbic acid (1mM) was also strongly inhibited by EDTA (0·2mM), inhibition being virtually complete in spleen and kidney homogenates and about 60% in heart and liver homogenates.

o-Phenanthroline or 8-hydroxyquinoline form complexes with inorganic iron that are very effective as catalysts of the peroxidation of unsaturated fatty acids (Wills, 1965). If non-haem iron is an important catalytic constituent of tissues it would therefore be expected that the addition of *o*-phenanthroline to tissue homogenates would accelerate the rate of peroxide formation. Addition of *o*-phenanthroline (1mM) did enhance the rate of peroxidation of linolenic acid emulsions catalysed by heart, spleen, kidney or liver homogenates but it strongly inhibited endogenous peroxide formation in incubated homogenates (Table 6). Similar effects were obtained with 8-hydroxyquinoline.

Effect of added haemoglobin. Haemoglobin and

Table 4. *Catalysis of the peroxidation of linoleic acid by tissue homogenates after treatment with hydrogen peroxide*

A 2ml. portion of tissue homogenate (10%, w/v) was kept at 50° for 5min. with 3% (w/v) H_2O_2 (0·4ml.). After cooling the rates of oxidation of linoleic acid emulsion catalysed by treated homogenates with and without ascorbic acid (1mM) were compared with that catalysed by control homogenates. Experimental details were as described in Table 2.

Tissue homogenate	Rate of peroxidation (μl. of O_2/min.) Control	H_2O_2-treated	H_2O_2-treated + ascorbic acid
Heart	2·81	0·32	1·36
Spleen	5·60	0·22	2·11
Kidney	1·47	0	4·00
Liver	3·02	0·18	4·44

Table 5. *Effect of EDTA and ascorbic acid on the rate of peroxidation of linolenic acid emulsion catalysed by tissue homogenates*

Experimental details were as described in Table 2.

Tissue homogenate	Rate of peroxidation (μl. of O_2/min.) Control	+EDTA (0·2mM)	+Ascorbic acid (1mM)	+EDTA (0·2mM) + ascorbic acid (1mM)
Heart	2·84	2·50	4·45	2·56
Spleen	5·38	4·89	5·92	5·20
Kidney	2·60	1·74	4·85	1·44
Liver	3·46	1·92	3·61	1·99

Table 6. *Effect of* o-*phenanthroline on lipid 'peroxide' formation in incubated tissue homogenates and on tissue catalysis of linolenic acid peroxidation*

Experimental details were as described in Table 2 except that linolenic acid emulsion was replaced by water for the study of endogenous 'peroxide' formation. O_2 uptake and thiobarbituric acid (TBA) values were measured during the study of linolenic acid peroxidation.

Tissue homogenate	o-Phenanthroline (1mM)	$10^3 \times$ Endogenous 'peroxide' formation in tissue homogenate as increase of TBA value (E_{535}/min.)	Peroxidation of linolenic acid: $10_3 \times$ Increase of TBA value (E_{535}/min.)	Peroxidation of linolenic acid: O_2 uptake (μl./min.)
Kidney	−	1·89	3·52	1·50
	+	0·86	13·11	5·58
Liver	−	2·05	5·78	3·45
	+	0·82	14·00	7·05

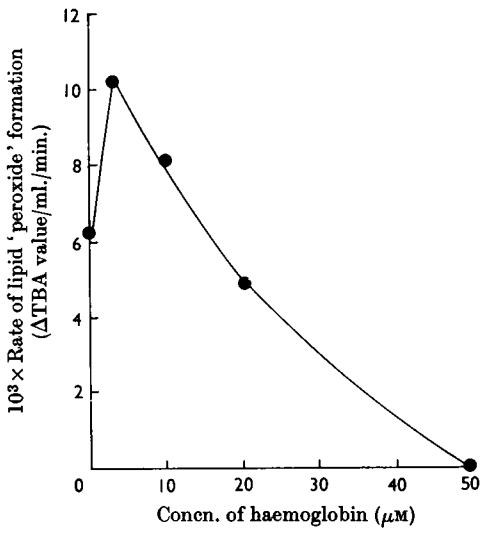

Fig. 4. Effect of haemoglobin on lipid 'peroxide' formation in incubated kidney homogenate. Kidney homogenate (5%, w/v) was suspended in 0·125M-KCl in 0·02M-phosphate buffer, pH6·4, and incubated at 37° in the presence of different concentrations of haemoglobin. Samples (0·5ml.) were removed periodically for determination of lipid 'peroxide' by the thiobarbituric acid method to give TBA values (as E_{535}).

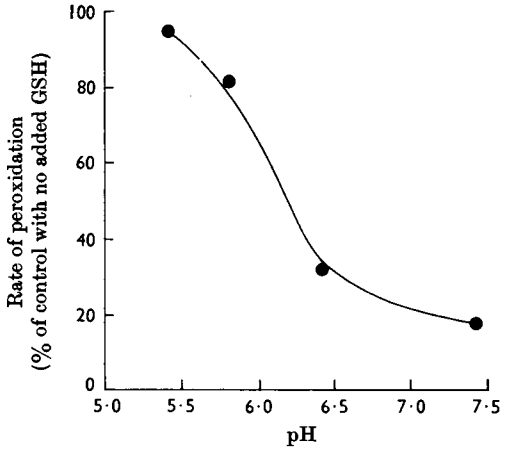

Fig. 5. Effect of pH on GSH inhibition of linoleic acid peroxidation catalysed by liver homogenate. Linoleic acid (16·7mM) in 0·02M-buffer (acetate, pH5·4, phosphate, pH5·8, phosphate, pH6·4, tris–HCl, pH7·4, or phosphate, pH7·4) was incubated with liver homogenate (final concn. 1%, w/v) for 60min. at 37°. Rates of peroxidation were measured in the presence and absence of GSH (2mM).

other haemoproteins are very active catalysts of lipid peroxide formation in solutions more dilute than 10μM but inhibit peroxide formation in more concentrated solutions (Lewis & Wills, 1963). Addition of haemoglobin to kidney homogenates increased endogenous lipid 'peroxide' formation if its concentration was not more than 10μM but completely inhibited 'peroxide' formation at concentrations above 50mM (Fig. 4). Results with liver and heart homogenates were essentially similar.

Effect of thiol compounds. Addition of GSH to homogenates (1%, w/v) of spleen, kidney, liver or heart caused inhibition of the peroxidation of linoleic acid or linolenic acid. Inhibition was observable with 0·2mM-GSH and was normally complete when the concentration of GSH was 1mM. If, however, the concentration of GSH was further increased (2–5mM) a rapid rate of lipid 'peroxide' formation occurred. The precise concentration of GSH required to cause inhibition or stimulation of the peroxidation varied with the tissue used and with the experimental conditions. The effect of GSH on the rate of peroxidation increased with increase of pH (Fig. 5). GSSG did not inhibit 'peroxide' formation.

Table 7. *Iron content of rat tissues*

Values are average determinations for ten animals.

Tissue	Iron (μmoles/g. wet wt. of tissue) Total iron	Non-haem iron	Haem iron
Heart	1·60	1·01	0·59
Spleen	8·11	4·79	3·32
Kidney	1·62	1·05	0·57
Liver	2·37	1·45	0·92

Iron content of tissues. All tissues contained both non-haem and haem iron (Table 7).

The very high concentration of haem iron in spleen is no doubt responsible for most of the catalytic activity observed with this tissue, whereas kidney catalysis probably depends considerably on non-haem iron. The high concentration of non-haem iron in spleen is mainly iron that is combined as ferritin or haemosiderin and is inactive catalytically. It is noteworthy that spleen-catalysed peroxidation is little stimulated by ascorbic acid whereas peroxidation catalysed by kidney homogenate is powerfully stimulated (Table 3).

Catalysis of peroxidation of unsaturated fatty acids by liver fractions. Little catalytic activity was associated with the nuclear fraction when the blood had been completely removed but the mitochondria, microsomes and final supernatant all possessed activity (Table 8). Ascorbic acid (1 mM) decreased 'peroxide' formation in the nuclear, mitochondrial and microsomal fractions to about 30% of the control at pH 7·4 but the catalytic activity of the supernatant fraction was increased by ascorbic acid (Table 8). *o*-Phenanthroline (1 mM) increased the catalytic activity of all fractions for the peroxidation of linoleic acid, whole liver 3·26-fold, mitochondria 3·47-fold and microsomes 2·32-fold, but the increase was especially marked in the supernatant fraction, which was 8·36 times as active as the control with no *o*-phenanthroline. The importance of cytochrome *c* in mitochondrial catalysis was established by decreasing the concentration of cytochrome *c* by the method of Jacobs & Sanadi (1960). This treatment decreased the rate of peroxidation of linoleic acid emulsion to 35% of that catalysed by normal mitochondria. GSH at a concentration of 2 mM inhibited mitochondrial catalysis of the peroxidation of linoleic acid and linolenic acid. However, with 5 mM-GSH a rapid rate of peroxidation ensued, as measured both by oxygen uptake and by the thiobarbituric acid method (Fig. 6). Peroxidation catalysed by 10 μM-cytochrome *c* was inhibited by 1 mM-GSH, and increasing the concentration of GSH caused increased inhibition (Fig. 6).

Table 8. *Catalysis of peroxidation of linoleic acid by liver fractions and the effect of ascorbic acid*

Liver fractions prepared from 8 g. of liver by the method of Aldridge (1957) were suspended in 8 ml. of 0·25 M-sucrose. Linoleic acid emulsion (0·02 M) in 0·25 M-sucrose (2·0 ml.), suspension of liver particles (0·3 ml.), 0·2 M-tris–HCl buffer, pH 7·4 (0·3 ml.), and 0·25 M-sucrose (0·2 ml.) were added to the flasks of Warburg manometers and the rates of O_2 uptake measured for 60 min. Rates are expressed in relation to the weight of liver used for the preparation.

Tissue fraction	Ascorbic acid (1 mM)	Rate of peroxidation (μl. of O_2/min./100 mg. of liver)
Whole homogenate	−	96
	+	33
Mitochondria	−	10
	+	3
Microsomes	−	32
	+	11
Supernatant (100000*g*)	−	34
	+	77

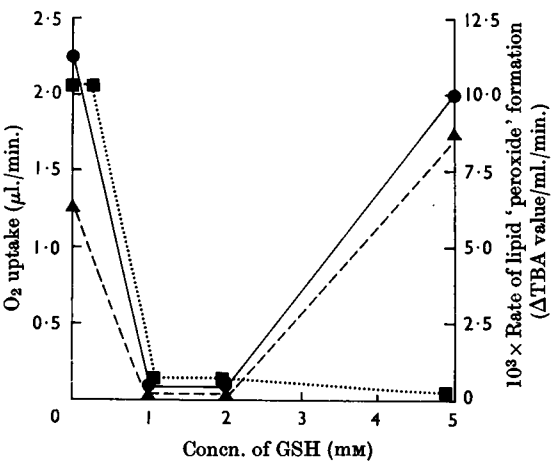

Fig. 6. Effect of GSH on the rate of linolenic acid peroxidation catalysed by liver mitochondria and by cytochrome *c*. Mitochondria prepared from 6 g. of liver were suspended in 6 ml. of 0·25 M-sucrose. Mitochondrial suspension (0·1 ml.) was added to 16·7 mM-linolenic acid in 0·02 M-tris–HCl buffer, pH 7·4, in a total volume of 3·0 ml. Rates of O_2 uptake were measured and thiobarbituric acid values were determined. Similar conditions were used for the oxidation catalysed by 10 μM-cytochrome *c*. Experiments were repeated in the presence of several different concentrations of GSH. Results of mitochondrial experiments are expressed in terms of O_2 uptake and of thiobarbituric acid (TBA) values (as E_{535}); cytochrome *c* experiments are recorded as O_2 uptake only. ●, Mitochondria (O_2 uptake); ▲, mitochondria (TBA values); ■, cytochrome *c*.

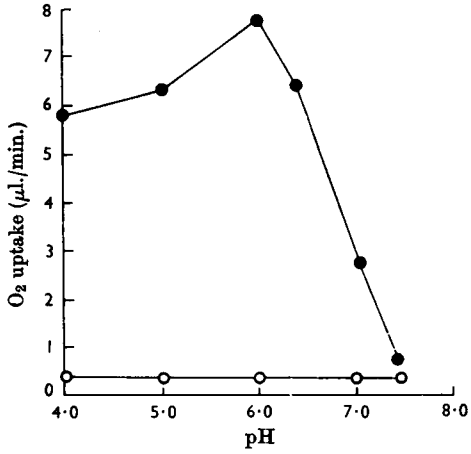

Fig. 7. Effect of ascorbic acid on ferritin-catalysed peroxidation of linoleic acid. A 0·1 ml. portion of 0·2% ferritin was added to 16·7 mM-linoleic acid in 0·02 M-buffer (acetate, pH 4·0, or phosphate, pH 6·0–8·0) in a total volume of 3·0 ml. The oxygen uptake at 37° was measured. Experiments were repeated in the presence of ascorbic acid (3·3 mM). ○, Ferritin; ●, ferritin + ascorbic acid.

Ferritin and fatty acid oxidation. A large proportion of tissue non-haem iron is stored in the form of ferritin and haemosiderin. This is especially true in liver and spleen. Ferritin (0·013%) did not increase the rate of peroxidation of linoleic acid emulsion in the range pH 4·0–7·4 but when ascorbic acid was added a rapid rate of peroxidation ensued. The rate was pH-dependent, and was most rapid at pH 6·0 (Fig. 7). Ascorbic acid is known to release iron from ferritin (Mazur, Baez & Shorr, 1955) and it has been shown that inorganic iron is an active catalyst on the acid side of neutrality (Wills, 1965).

DISCUSSION

Reliable analysis of peroxides *in vivo* is difficult (Philpot, 1963), and therefore the question as to whether lipid peroxides are formed *in vivo* is not yet resolved. Nevertheless isolated tissue always contains some 'peroxide' (Zalkin & Tappel, 1960; Bieri & Anderson, 1960). Further, 'peroxides' are very readily formed *in vitro* in tissues removed from animals, and it therefore seems likely that, under certain conditions, peroxide formation can occur *in vivo*.

Peroxide formation *in vivo* would produce serious consequences. First, since it is well known that peroxide formation in unsaturated fatty acids results in rupture of the carbon chain, it is likely that disintegration of essential structural components, such as lipoprotein membranes, would result. Leakage of lysosomal enzymes has been associated with lipid peroxide formation in lysosomes (Wills & Wilkinson, 1966). Secondly, peroxides may have toxic effects on vital cell constituents. For example, lipid peroxides can inactivate thiol enzymes (Wills, 1961) and oxidize thiol groups of amino acids, peptides and proteins (Lewis & Wills, 1962). In addition, it is possible that hydroperoxide formation could constitute an important metabolic pathway; thus it has been proposed that the hydroperoxide of cholesterol linoleate is responsible for the introduction of a hydroxyl group into the sterol ring (Boyd, 1962).

The present experiments demonstrate that the tissues studied, liver, kidney, spleen and heart, can all form lipid 'peroxides', as measured by the thiobarbituric acid method, when incubated *in vitro*. All the tissues also actively catalyse the peroxidation of added unsaturated fatty acids such as linoleic acid, and linolenic acid. Incubated brain homogenate also forms 'peroxides' readily (Barber, 1963).

In nearly all work previously reported determinations of lipid peroxide have been carried out by the thiobarbituric acid method. This method can measure the progress of oxidation of linolenic acid and fatty acids with a higher degree of unsaturation but not of linoleic acid (Wilbur *et al.* 1949). It may be unreliable under some other conditions. When tissue homogenates catalyse the oxidation of linolenic acid an increase of 1 unit in the thiobarbituric acid value is accompanied by an oxygen uptake of about 0·89 μmole/ml. As described in the Results section, this quantity is less than the concentration of 'peroxide' that was determined by the thiobarbituric acid method, namely 1·4 μmoles of peroxide/ml. There are two possible explanations for this. First, there are difficulties in the determination of the molar extinction coefficient of peroxide at 232 mμ (Mead, 1952), and, secondly, it is likely that partially oxidized fatty acids in tissue homogenates could break down without further oxygen uptake to give fragments that are determined by the thiobarbituric acid method. Experiments with ascorbic acid (Table 3) indicate the possible extent to which thiobarbituric acid values can increase out of proportion to oxygen uptake.

Heart, kidney and liver homogenates formed endogenous 'peroxide', as measured by the thiobarbituric acid method, and to a reasonable approximation their catalytic activity for linolenic acid peroxidation paralleled the endogenous 'peroxide' formation. Of these three tissues liver was most active and heart last. Spleen, however, was exceptional. It formed little endogenous 'peroxide', but of the tissues used it was the most active tissue for the peroxidation of added linolenic

acid. Measurements of endogenous 'peroxide' formation may therefore be unreliable as an indication of the capability of a tissue for catalysis of peroxide formation.

There was poor agreement between increase of oxygen uptake and thiobarbituric acid values produced by the addition of ascorbic acid, the increase of thiobarbituric acid value always being considerably more than the corresponding increase in oxygen uptake (Table 3). It is possible that ascorbic acid catalyses the split of malonaldehyde or other component of the peroxidized fatty acids that reacts in the thiobarbituric acid method of determination and that the total increase in thiobarbituric acid value observed when ascorbic acid is added is not due to genuine peroxide formation.

The enhanced rate of lipid 'peroxide' formation observed when ascorbic acid is added to tissue homogenates may arise from the catalytic system formed from non-haem iron and ascorbic acid. Alternatively, ascorbate in the presence of a heavy metal such as iron could form hydrogen peroxide, a process that may initiate a rapid rate of peroxidation possibly through the intermediary formation of hydroxyl radicals. The iron content of each tissue, and whether the iron is in the haem form, clearly has an important effect on catalysis. Spleen has the greatest content of haem iron (Table 7) and, although the quantity of non-haem iron is high, this iron is probably mainly as ferritin and haemosiderin and therefore unavailable for catalysis. Catalysis by spleen homogenate is probably mainly a result of haemoprotein activity. In support of this Fig. 1 shows that high concentrations of spleen homogenate tend to inhibit peroxidation of fatty acids exactly as haemoproteins do (Lewis & Wills, 1963), and catalysis by spleen homogenate is very little affected by ascorbic acid. On the other hand catalysis in kidney homogenates depends, to a large extent, on the relatively large quantity of non-haem iron, and catalysis by kidney homogenate is strongly stimulated by ascorbic acid (Table 3). Although purified ferritin is ineffective as a catalyst, the iron can be released by ascorbic acid to become an effective catalyst. Whenever ferritin is present in the tissue ascorbic acid can therefore perform a dual role, first in releasing iron from the ferritin and secondly by forming an active catalytic system with the iron.

At pH 6·0 the rate of peroxidation of linoleic acid or linolenic acid increased with increasing homogenate concentration up to a specific concentration of homogenate. Further increases in concentration of homogenate did not increase the rate of peroxidation (Fig. 1). This concentration effect is pH-dependent and concentrated homogenates are very effective catalysts in acid solutions but very weak catalysts on the alkaline side of neutrality (Figs. 2 and 3). It has been shown (Wills, 1965) that catalysis of lipid peroxide formation by inorganic iron is pH-dependent, the optimum rate of peroxidation being close to pH 5·5, and catalytic activity is weak when the reaction mixture is more alkaline than pH 7·0. It is therefore likely that a major portion of the catalytic activity of homogenates in acid solutions is due to non-haem iron but as the reaction is made more alkaline the proportion of total catalysis due to haemoproteins increases. If the homogenate concentration is increased in an alkaline solution then the inhibiting effects of high concentrations of haemoproteins become apparent.

Several other experiments demonstrate the complex roles of inorganic iron (non-haem) and haemoproteins in peroxide formation. EDTA powerfully inhibits iron-catalysed peroxidation of unsaturated fatty acids but has no effect on haemoprotein-catalysed oxidation (Wills, 1965). EDTA partially inhibited peroxide formation in added linolenic acid catalysed by liver or kidney homogenates but had little effect on catalysis by heart or spleen homogenates (Table 5). In these latter tissues it is therefore likely that haemoproteins are of major importance in the catalysis, whereas non-haem iron is at least as important as haemoproteins in kidney or liver. Effects of chelating agents on endogenous 'peroxide' formation are often different from their effects on the peroxidation of added unsaturated fatty acids. For example, endogenous 'peroxide' formation in spleen and kidney is powerfully inhibited by EDTA but peroxidation of added linolenic acid was much less affected. From these experiments it may be inferred that endogenous 'peroxide' formation is catalysed mainly by non-haem iron, or other metal, contained in the tissues, whereas haemoproteins catalyse the peroxidation of added linolenic acid. The effect of *o*-phenanthroline is difficult to interpret. The rate of peroxidation of added fatty acids is strongly accelerated by the addition of *o*-phenanthroline, and this is presumably due to the formation of an iron–*o*-phenanthroline complex. The inhibition of endogenous 'peroxide' formation by *o*-phenanthroline is in direct contrast with its enhancing effect on the rate of peroxidation of added fatty acid emulsions. It has been shown (Wills, 1965) that *o*-phenanthroline inhibits cobalt-catalysed peroxidation in model systems and it is therefore possible that a metal other than iron is partially or primarily responsible for 'peroxide' formation in incubated homogenates.

Thiol compounds, cysteine and GSH, accelerate catalysis of peroxide formation by inorganic iron (Wills, 1965) but delay haemoprotein-catalysed peroxidation (Lewis & Wills, 1963; Wills, 1965). GSH at a concentration of 1 mM inhibited the

peroxidation catalysed by homogenates but it became an activator at concentrations greater than 2mM. Catalysis by cytochrome *c* was, under similar conditions, inhibited by GSH at this high concentration. It therefore appears that low concentrations of GSH inhibit haemoprotein-catalysed peroxidation, whereas higher concentrations cause an increase in peroxidation catalysed by non-haem iron.

All fractions of liver studied possessed catalytic activity for the peroxidation of unsaturated fatty acids but little activity was associated with the nuclear fraction. The effects of chelating agents, ascorbic acid and thiol compounds indicated that in most fractions catalysis by haemoprotein and non-haem iron are both important. In the mitochondrial and microsomal fraction, haemoproteins are likely to be more important because ascorbic acid inhibited peroxidation of fatty acids and, further, decrease of the cytochrome *c* content of the mitochondria considerably diminished the catalytic activity. Ascorbic acid strongly stimulated catalysis by the 100000*g* supernatant and presumably non-haem iron is important in this fraction.

Most of the experiments described therefore implicate both non-haem iron and haemoproteins in the catalysis of lipid peroxide formation. Stored iron in the form of ferritin cannot catalyse lipid peroxide formation but iron released in an inorganic (non-haem) form or converted into haemoprotein is active catalytically. Ascorbic acid releases iron from ferritin. Inorganic iron either as Fe^{2+} or Fe^{3+} is a very weak catalyst unless activated by a reducing agent (Wills, 1965): either ascorbic acid or thiol compounds can function in this role. Iron-catalysed oxidation is pH-sensitive and most active in acid conditions. Haemoprotein-catalysed oxidation on the other hand is little affected by pH and is inhibited, at least temporarily, by reducing agents such as ascorbic acid or thiol compounds.

Lipid peroxide formation in tissues will also depend on the tissue content of tocopherol and other antioxidants (Bieri & Anderson, 1960; Zalkin & Tappel, 1960) but no attempt has been made to vary the dietary and therefore the concentration of antioxidant in the tissues in the present investigation.

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