Biochemical Pharmacology, 1962, Vol. 11, pp. 901–912. Pergamon Press Ltd., Printed in Great Britain.

THE DESTRUCTION OF —SH GROUPS OF PROTEINS AND AMINO ACIDS BY PEROXIDES OF UNSATURATED FATTY ACIDS

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(*Received* 26 *April* 1962; *accepted* 13 *June* 1962)

Abstract—Emulsions of linoleic acid and other unsaturated fatty acids, when incubated at 37 °C in air with cysteine, glutathione and sulphydryl proteins such as papain, cause a rapid destruction of —SH groups. The rate of —SH group destruction is slowed down in a nitrogen atmosphere but considerably increased in oxygen. Sulphydryl groups are destroyed much more rapidly by oxidized linoleic acid emulsions than by fresh emulsions and the rate of destruction is proportional to the peroxide value of the emulsion. During the destruction of —SH groups by fatty acid peroxides the peroxides themselves are destroyed. The exact fate of the —SH groups is uncertain but cysteine has been shown to be converted to a mixture of cystine, cysteic acid and cystine disulphoxide.

INTRODUCTION

UNSATURATED fatty acids are of widespread occurrence in animal and plant tissues. It has been known for a considerable time that these fatty acids autoxidize readily *in vitro* to form peroxides,[1, 2] but the importance of oxidation *in vivo* is not yet fully established. Nevertheless, the widely held view that oxidation of unsaturated fatty acids does not occur *in vivo* because of the protective effect of vitamin E has not been substantiated by more recent work.[3] Peroxides have been shown to be formed in red cells under certain conditions[4, 5] and in mitochondria,[6] but probably the most interesting discovery is the formation of lipid peroxides after irradiation of plants[7] and of the presence of unknown peroxides in irradiated animals.[8]

Certain limited studies of the biochemical effects of peroxides have been carried out. Horgan *et al.*[9] found that peroxides of linoleic acid were toxic when injected into mice. Dubouloz and Fondarai[10] showed that ethyl oleate peroxide could oxidize the—SH groups of protein to —S—S—, and Schauenstein *et al.*[11] found that the hydroperoxide of linoleic ester caused the destruction of —SH groups of cysteine, but the studies were made under a limited range of experimental conditions. Bernheim *et al.*[12] found that oxidized unsaturated fatty acids were toxic to certain oxidative enzymes, but Wills[13] found that the toxicity of peroxides of unsaturated fatty acids was not confined to oxidative enzymes but was a more general phenomenon and that —SH enzymes were especially sensitive.

In view of these findings it was decided that it would be desirable to investigate the effect of fatty acid peroxides on —SH compounds of different types and under different conditions. It has been found that all —SH compounds are attacked by fatty acid peroxides but that the effect depends on the peroxide value of the fatty acid

used, the time of incubation and on the temperature. The destruction of —SH groups is an oxidative process but does not occur instantaneously.

MATERIALS AND METHODS

Materials

Palmitic acid was obtained from British Drug Houses, Ltd., and pure linoleic acid from California Research Foundation and from British Drug Houses, Ltd. Linolenic acid was obtained from B.D.H. Ltd.

Cysteine was obtained from B.D.H. Ltd. and glutathione from B.D.H. Ltd. and L. Light & Co. Ltd. Determination of —SH groups showed that both preparations contained identical quantities of reduced —SH groups and were nearly 100 per cent pure.

Crude papain was obtained from Hopkin & Williams and haemoglobin from Armour Ltd.

All water used in the preparation of solutions used in the experiments had been passed through a de-ionising column and then distilled from alkaline $KMnO_4$ in an all glass still. Emulsions of fatty acids were prepared by diluting ethanolic solutions of fatty acids with phosphate buffer;[14] the final concentration of phosphate buffer was $2{\cdot}5 \times 10^{-2}$ M and the ethanol was 10% (v/v). The concentration of linoleic acid usually varied between 10^{-2} M and 2×10^{-3} M. Emulsions were prepared immediately before use in the majority of experiments.

Determination of —SH *groups*

Sulphydryl groups were determined by two methods; by the iodate titration method of Woodward and Fry[15] and by an amperometric method which incorporated the modifications described by Thomson and Martin[16] of the original method of Benesch *et al.*[17] The iodate method was used for determination of —SH compounds when the concentration of —SH groups fell between 4 and 40 μmole SH/ml. The amperometric method could be used for samples from twenty to a hundred times less concentrated than those used for the iodate titration, and in most experiments the concentration of —SH groups was 0·03–0·5 μmole/0·5 ml sample.

Determination of peroxide content of emulsions

In most experiments the peroxide values were determined by the ferric thiocyanate method as described by Wagner *et al.*[18] This method is not the most accurate but is quick and convenient for comparative work. The sample (0·4 ml) was added to 10 ml reagent and the red colour produced measured in the EEL Colorimeter using filter no. OB10, and compared with standard ferric chloride treated similarly.

In certain experiments when linolenic acid was used peroxide values were determined on 1-ml samples, by the thiobarbituric acid colorimetric method following the technique described by Wilbur *et al.*[19] In a few cases the oxidation of the linoleic acid was measured by determining the absorption at 235 mμ.[20]

The results were compared by two methods whenever possible.

RESULTS

Effect of emulsions of linoleic acid and palmitic acid on cysteine and glutathione

In a typical experiment 2×10^{-2} M linoleic acid emulsion (1·0 ml), 0·25 M pH 7·4 phosphate buffer (1·0 ml) were mixed in an open conical flask which was then shaken

in a water bath at 37 °C. As soon as the contents of the flask had reached 37 °C, 10^{-2} M cysteine (1·0 ml) was added, a sample (0·5 ml) was removed and added to 0·25 ml of sulphosalicyclic acid (4% w/v). The mixture was then added to the Tris buffer and titrated with 10^{-3} N silver nitrate. Samples were removed at 10–20 min intervals for 90 min or longer. The addition of sulphosalicylic acid caused the pH of the sample to fall to 2·0 and when the mixture was kept at 5° there was no significant loss of —SH groups over a 5-hr period. This procedure was convenient if the titration could not be performed immediately.

A control experiment in which the linoleic acid emulsion was replaced by $2{\cdot}5 \times 10^{-2}$ M of pH 7·4 phosphate buffer (1·0 ml) containing 10% ethanol was carried out concurrently with every experiment utilizing an emulsion.

The experimental procedure was repeated using 10^{-2} M glutathione (1·0 ml) in place of the cysteine.

During the course of the experiment the concentration of —SH groups in the control experiment without linoleic acid showed a slow but steady fall and this rate was increased by the presence of linoleic acid emulsion. If the linoleic acid was replaced by palmitic acid emulsion there was no alteration in the rate of —SH group destruction. When cysteine was replaced by glutathione addition of linoleic acid emulsion speeded the rate of —SH group destruction but to a smaller extent than was observed when using cysteine.

The effect of air, oxygen and nitrogen on the rate of destruction of —SH *groups by linoleic acid emulsion*

Sulphydryl groups are likely to be destroyed by one of two processes, either (a) by an addition to the double bonds of the unsaturated fatty acids,[21] or (b) by oxidation to —S—S and other oxidation products which is caused by the development of peroxides at the double bonds of fatty acids. A simple distinction between the two possibilities may be made by comparing the rate of —SH group destruction in oxygen and in nitrogen. If process (a) were of major importance it would be expected that there would be little difference in rate in either oxygen or in nitrogen, but if the oxidation at the double bonds of the fatty acid was an essential part of the process, then the rate of —SH group destruction in oxygen would be much faster. In order to determine the relative importance of these mechanisms, 2×10^{-2} M cysteine (1 ml), $5{\cdot}2 \times 10^{-3}$ M linoleic acid emulsion (from 1·0 ml to 9·0 ml) in phosphate buffer pH 7·4 were mixed and made up to 10 ml with water in small corked conical flasks. Three experiments were normally carried out simultaneously. One flask was gassed with oxygen, another with nitrogen and the third contained air. All flasks were set shaking at 37 °C in a water bath. Samples (1 ml) were withdrawn at 15 min intervals and the —SH group concentration determined by the iodate method. A series of experiments of this type was carried out in which the linoleic acid concentration was varied between 0 and 5×10^{-3} M. The rate of —SH group destruction was found to be very rapid in oxygen and very slow in nitrogen (Fig. 1), a fact which clearly indicates that oxidation is an important factor in the destructive process.

The effect of oxidized linoleic acid emulsions on the rate of destruction of —SH *groups*

Early in the work it soon became apparent that linoleic acid emulsions of the same

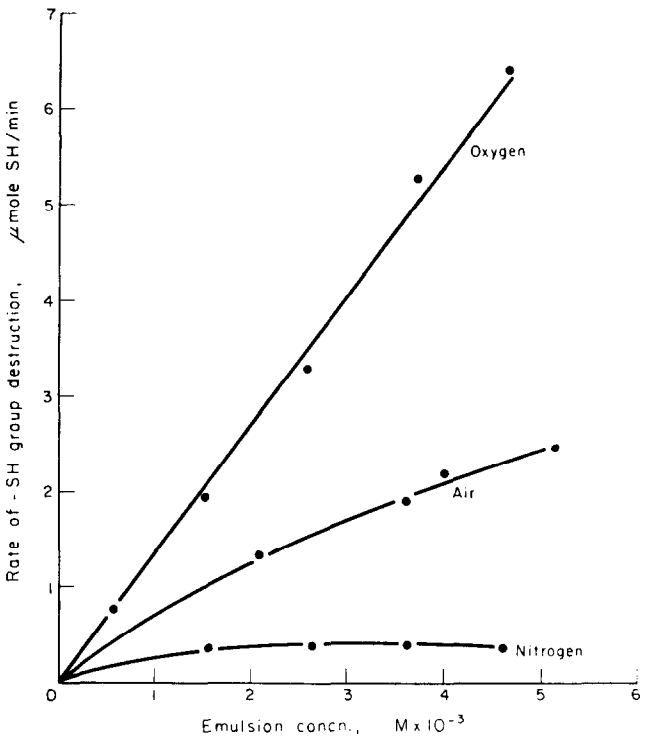

FIG. 1. Comparison of the effects of oxygen, nitrogen and air on the rate of destruction of —SH groups of cysteine, (2×10^{-2} M) by linoleic acid emulsion.

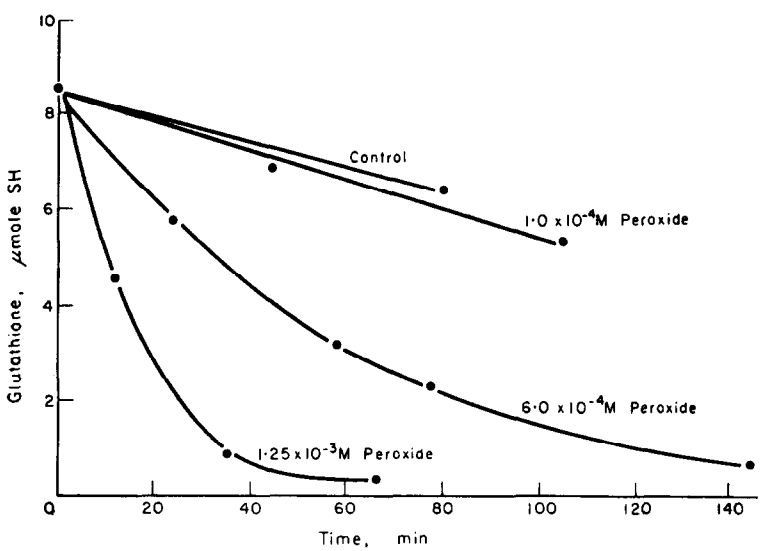

FIG. 2. Effect of linoleic acid emulsions at various stages of oxidation on the rate of —SH group destruction of glutathione. 10^{-3} M glutathione, 2×10^{-3} M linoleic acid emulsion.

concentration did not destroy —SH groups at the same rate. Fresh emulsions were least effective and emulsions which had been allowed to stand were more effective.

The effect of oxidized emulsions on —SH group destruction was investigated by incubating 2×10^{-2} M linoleic acid in air at 37 °C for periods which varied between 2 and 24 hr. The peroxide value of each emulsion was determined by the ferric thiocyanate method and then these oxidized emulsions were incubated with glutathione. Glutathione 10^{-2} M (1·0 ml) was mixedwith 2×10^{-2} M oxidized linoleic acid emulsion (1·0 ml), 0·25 M pH 7·4 phosphate buffer (1·0 ml), made up to 10 ml with water, and the mixture incubated at 37 °C. Samples (0·5 ml) were removed at 10–20 min intervals for 2 hr. The experiment was repeated using several emulsions of different peroxide content. It was found that the greater the peroxide value of the emulsion, the greater the rate of —SH group destruction (Fig. 2).

The experiment was repeated using cysteine and an extract of crude papain (0·5%). In each case the rate of destruction of —SH groups was directly proportional to the peroxide value of the emulsion (Fig. 3).

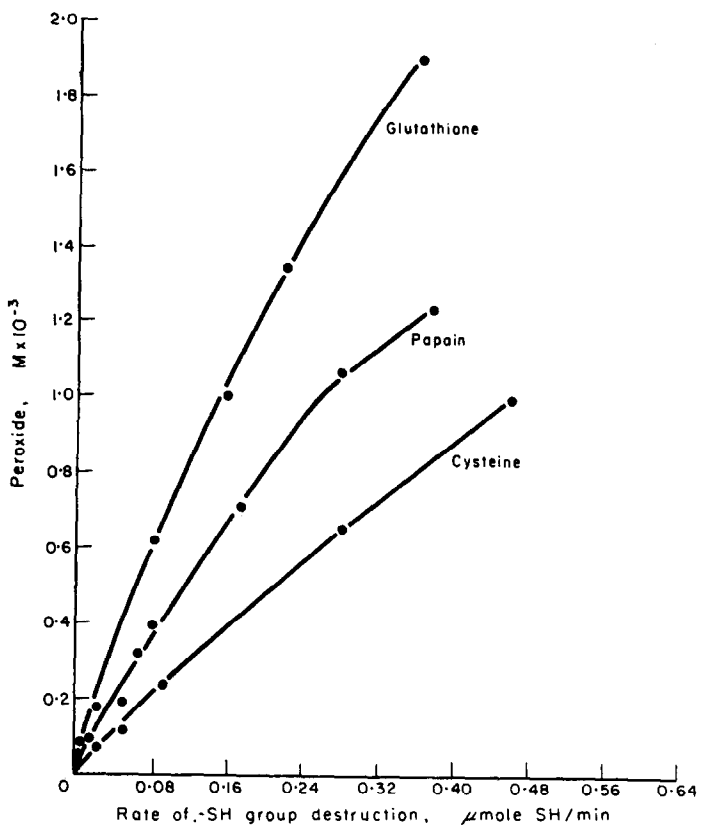

FIG. 3. Comparison of the rates of —SH group destruction of cysteine, glutathione and papain by oxidized linoleic acid. 10^{-3} M cysteine, 10^{-3} M glutathione, 0·5% papain (crude enzyme), and 2×10^{-3} M linoleic acid emulsion.

Effect of oxidized linoleic acid emulsions on —SH *groups in a nitrogen atmosphere*

Linoleic acid emulsion ($5{\cdot}2 \times 10^{-3}$ M) was allowed to oxidize for 48 hr and ultimately contained $2{\cdot}6 \times 10^{-3}$ M peroxide oxygen. Cysteine, 0·2 M (1 ml) was incubated with the oxidized linoleic acid emulsion in 5×10^{-2} M pH 7·4 phosphate buffer in an atmosphere of nitrogen. Several different volumes of this emulsion were used and these ranged between 9·0 ml and 1·0 ml. The total volume was made up to 10 ml with water. Samples (1·0 ml) were taken at 10-min intervals and the —SH concentration was determined by the iodate method. The concentration of —SH groups fell rapidly for about 30 min after which it did not change for at least 2 hr but the rate of disappearance of —SH groups in nitrogen was only about a third of the rate of —SH group destruction in air. The fall of —SH concentration was found to be proportional to the concentration of peroxide in the oxidized linoleic acid. By carrying out the experiment in nitrogen no additional peroxide could be formed and the destruction of —SH groups could only result from added peroxide.

The fate of —SH *compounds after treatment with oxidized linoleic acid emulsions*

In order to attempt to determine more precisely the fate of —SH groups after oxidation by oxidized linoleic acid emulsions, 2×10^{-2} M oxidized linoleic acid emulsion containing $2{\cdot}8 \times 10^{-3}$ M peroxide (4 ml) was incubated with 0·1 M cysteine (0·5 ml) and pH 7·4, 0·25 M phosphate buffer (0·5 ml) at 37 °C for 3 hr. These experimental conditions had been previously found to cause complete destruction of cysteine —SH groups. At the end of the incubation, samples of the mixture were examined chromatographically using standard solvents, e.g. *n*-butanol–acetic acid and *tert.*-butanol–ethylmethyl ketone–diethylamine in a two dimensional system, but only a poor separation of the cysteine derivatives could be achieved, on account of the close similarity of the R_F values of cysteine oxidation products. Good separation was, however, obtained with a one way run using 100 ml *n*-butanol, 100 ml ethylmethylketone, 20 ml *dicyclo*hexylamine and 47 ml water as solvent as suggested by Bowden[22]. Cystine, cysteic acid and some cystine disulphoxide could be recognized after incubation of the cysteine with oxidized linoleic acid emulsion. Cysteine incubated alone formed a small quantity of cystine only.

Fate of peroxide after incubation with —SH *compounds*

In order to study the fate of peroxide during —SH group oxidation it was necessary to measure peroxide values and —SH concentration simultaneously.

Oxidized linoleic acid emulsion 2×10^{-2} M (1·0 ml) containing $1{\cdot}3 \times 10^{-2}$ M peroxide oxygen was mixed with 10^{-2} M glutathione (1·0 ml) and 0·25 M pH 7·4 phosphate buffer (1·0 ml) in a conical flask and the volume made up to 10 ml. The mixture was incubated at 37 °C. Samples (0·5 ml) were removed immediately for amperometric determination of —SH groups and samples (0·4 ml) removed for determinations of peroxide values. The experiment was continued for 90 min. During the first 60 min of the incubation the —SH group concentration fell to zero and there was a fall in peroxide value of the emulsion but when all the glutathione had been oxidized the peroxide concentration began to rise again (Fig. 4). When the experiment was repeated substituting 10^{-2} M cysteine (1·0 ml) for glutathione, it was found that there was an even greater rate of fall of the peroxide value of the emulsion and that the cysteine was destroyed more rapidly than the glutathione. After all the cysteine had been destroyed the peroxide content of the emulsion increased.

The u.v. absorption at 235 mμ of oxidized linoleic acid emulsion (2×10^{-3} M) decreased in the presence of glutathione (10^{-3} M) but there was a steady increase of the u.v. absorption of a similar emulsion incubated at 37 °C without glutathione.

Cysteine (10^{-3} M), added to oxidized linoleic acid emulsion containing $6{\cdot}7 \times 10^{-4}$ M peroxide oxygen caused less destruction of peroxide as determined by the ferric

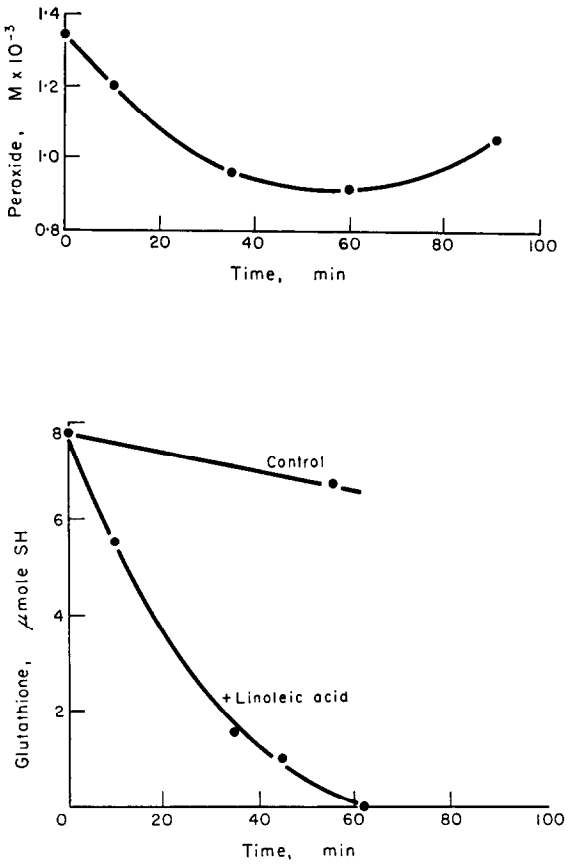

FIG. 4. Relationship between the peroxide value of linoleic acid and the rate of destruction of —SH groups of glutathione. 10^{-3} M glutathione, 2×10^{-3} M linoleic acid.

thiocyanate method than when added to an equivalent quantity of linoleic acid emulsion. When the experiment was repeated, determining peroxide by the thiobarbituric acid method, the peroxide concentration decreased to a small extent for a 30-min period and then increased again at a rate which was less than that of a control linolenic acid emulsion. The general pattern of behavior of linolenic acid was similar to that of linoleic acid and quantitative differences observed may be a result of the fact that linolenic acid emulsion oxidizes more rapidly than linoleic acid emulsion. Different methods of peroxide determination gave similar qualitative results but somewhat different quantitative results, presumably because entirely different types of measurement are made.

Effect of temperature on the rate of —SH *group destruction*

All experiments described in this work so far were carried out at 37 °C. If linoleic acid emulsion was incubated with glutathione or cysteine at 20 °C instead of at 37 °C, the rate of disappearance of —SH groups was very much slower. Thus, for example, when 2×10^{-3} M linoleic acid emulsion containing 10^{-3} M peroxide oxygen was incubated with 10^{-3} M cysteine at 37 °C the rate of destruction of —SH groups was 0·46 μmole/min but if the same experiment was carried out at 20 °C the rate was only 0·068 μmole/min. Comparable figures using glutathione were 0·16 μmole —SH group/min at 37 °C and 0·044 μmole —SH group/min at 20 °C. Determinations were carried out amperometrically and the rates are corrected for the rate of destruction of the sulphydryl compound alone.

Effect of haemoglobin and haemin on the destruction of —SH *groups by linoleic acid emulsions*

Haemoglobin and haemin have long been known to be very effective catalysts of the oxidation of unsaturated fatty acids[23, 24] but it has been recently demonstrated that in concentrated solutions both haemoglobin and haemin inhibit autoxidation.[14] It would

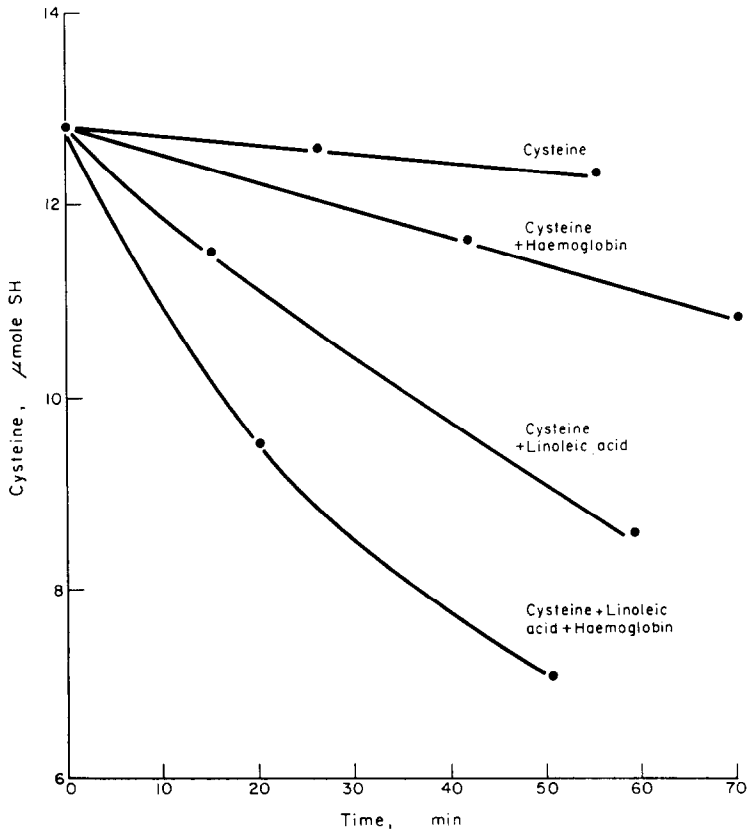

FIG. 5. Destruction of the —SH groups of cysteine by linoleic acid and haemoglobin. 10^{-3} M cysteine, 2×10^{-3} M linoleic acid emulsion, $2{\cdot}5 \times 10^{-6}$ M haemoglobin.

be expected that addition of haemoglobin or haemin to emulsions and sulphydryl compounds would speed up the rate of —SH group destruction.

Fresh linoleic acid emulsion (2×10^{-2} M), (1 ml), 10^{-2} M cysteine (1 ml) and 10^{-5} M haemoglobin (2·5 ml), 0·25 M pH 7·4 phosphate buffer (2·0 ml) were mixed, made up to 10 ml with water and incubated at 37 °C. Similar experiments were set up using cysteine alone, cysteine plus haemoglobin and cysteine plus linoleic acid. Samples (0·5 ml) were removed at intervals for the amperometric determination of —SH group concentration. Haemoglobin, alone, catalysed the oxidation of —SH groups but in the presence of linoleic acid the rate increased considerably (Fig. 5).

Haemoglobin was replaced by haemin (10^{-5} M) in this series of experiments and the concentration of —SH groups and peroxide content measured in each flask for a period of 4 hr.

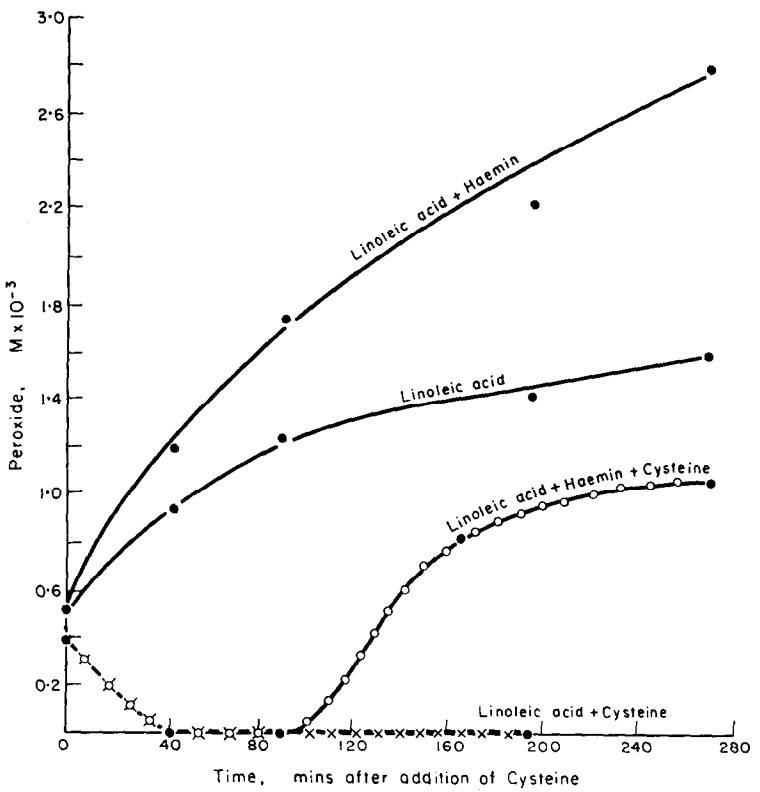

FIG. 6. Effect of haemin and cysteine on the rate of formation of peroxide by linoleic acid emulsion. 2×10^{-2} M cysteine, 10^{-5} M haemin and $6{\cdot}7 \times 10^{-3}$ M linoleic acid emulsion.

Haemin catalysed the oxidation of the linoleic acid emulsion, but the addition of cysteine abolished the oxidation and caused a decrease in the peroxide value for 90 min when all the cysteine had been destroyed. After this period, the rate of oxidation catalysed by haemin was similar to that of the experiment to which no cysteine was added (Fig. 6). Similar experiments were carried out using haemoglobin instead of

haemin and the rate of oxidation of linoleic acid determined manometrically. Cysteine was found to inhibit the oxygen uptake and oxidation of linoleic acid did not begin until all the cysteine had been destroyed, the delay in the start of oxidation depending on the concentration of cysteine added.[14]

When haemoglobin was incubated with linoleic acid emulsions and glutathione the effect on the rate of —SH group destruction was found to be dependent on the ratio of haemoglobin concentration to the emulsion concentration.

Linoleic acid (2×10^{-2} M) was mixed with glutathione (10^{-3} M) and phosphate buffer $2{\cdot}5 \times 10^{-2}$ M and the total volume made up to 3·0 ml. Different volumes of haemoglobin solution ($2{\cdot}5 \times 10^{-5}$ M) were added so that the final concentration of haemoglobin was varied between 10^{-5} M and $2{\cdot}5 \times 10^{-7}$ M. The mixture was incubated at 37 °C and samples were withdrawn for the determination of —SH groups amperometrically. When dilute solutions of haemoglobin, e.g. $2{\cdot}5 \times 10^{-6}$ M were used there was a significant increase in the rate of SH group destruction. This effect was dependent on the ratio of molar concentration of linoleic acid: molar concentration of haemoglobin. If the ratio fell between 200 : 1 to 8000 : 1 there was no effect on the rate of —SH group destruction but if more dilute haemoglobin was used the rate was significantly increased. Haemoglobin added in a concentration of 1 mole: 10,000 moles linoleic acid increased the rate 2·4 times and in a concentration 1 mole: 20,000 moles linoleic acid increased the rate 1·4 times. The catalytic effect of haemoglobin is discussed by Lewis and Wills[14].

DISCUSSION

Sulphydryl compounds such as glutathione and cysteine are not stable at pH 7·4 at 37 °C on account of a slow atmospheric oxidation of —SH groups to disulphides. These experimental conditions were, however, selected for the major portion of this work because they are close to the physiological. When emulsions of unsaturated fatty acids are incubated with cysteine or glutathione, the rate of —SH group destruction is considerably increased. The destruction process is primarily an oxidative one and there is very little disappearance of —SH groups of the sulphydryl compound if the incubation is carried out in nitrogen. The rate of —SH group destruction by unsaturated fatty acids may be considerably increased by (a) incubating the fresh emulsion with —SH compound in air or oxygen, (b) by first oxidizing the emulsion in air before adding the —SH compound, or (c) by adding a catalyst of oxidation of unsaturated fatty acids, e.g. haemoglobin. When fresh emulsions are incubated with —SH compounds in air or oxygen the destruction of —SH groups which occurs is mediated by the intermediate formation of fatty acid peroxides which oxidize the —SH groups. The rate of —SH group destruction by unsaturated fatty acids is considerably increased by prior incubation of the fatty acids in air or oxygen. This gives rise to hydroperoxide groups (—OOH) on carbon atoms adjacent to double bonds of the fatty acids. These peroxides cause a rapid destruction of —SH groups and the rate of disappearance of —SH groups is proportional to the peroxide content of the emulsion (Fig. 3). Oxidized unsaturated fatty acids also destroy —SH groups in a nitrogen atmosphere but the rate is slower than in air and ceases immediately the peroxide has been utilized in the oxidation. The rate of destruction of —SH groups was found to depend on the temperature and to be much less at 20 °C than at 37 °C and to be speeded up by catalysts of peroxide formation, e.g. haemoglobin or haemin (Fig. 5).

A chromatographic study of the products of cysteine after oxidation by fatty acid peroxides showed that in addition to the disulphide, cysteic acid and cystine disulphoxide were formed. This pattern of oxidation of —SH groups to —S—S—; —SO_3 and —S—S— is believed to occur at SH groups of other compounds including proteins and enzymes. In most respects the effects of fatty acid peroxides on cysteine and glutathione resemble those of fatty acid peroxides and of organic peroxides on sulphydryl enzymes.[13, 25] It is significant that it was concluded that peroxides oxidized —SH groups of enzymes to products other than the disulphides because of the inability of cysteine to reverse the inhibition.[25]

During the course of the oxidation of —SH groups, peroxides are destroyed but peroxides increase again on further incubation in the presence of the oxidation products of the sulphydryl compounds.

The biological implications of these findings may be far reaching. Wherever the ubiquitous unsaturated fatty acids are found in cells and in cell membranes, there will be the possibility of peroxide formation and thus of —SH destruction. The originally widely held view that all unsaturated fatty acids are adequately protected by vitamin E against oxidation under all conditions is not substantiated by more recent work.[3] There are many metals and metal containing compounds in cells which can act as powerful catalysts of oxidation of unsaturated fatty acids and catalysis is fully discussed by Lewis and Wills.[14] It has been shown that oxidation of unsaturated fatty acids results in changes in the permeability in the red cell membrane[4, 26] which in turn leads to the escape of haemoglobin from the cell. If such permeability changes occur as a result of oxidation of unsaturated fatty acids of intracellular membranes, catalysts of oxidation, e.g. haem-type proteins, may be released within the cell and effect oxidation of unsaturated fatty acids at new sites within the cell. Lack of anti-oxidant at special sites would accelerate this process.

Lipid peroxides have been demonstrated in plants[7] and animals[8] after irradiation and their formation may be connected with permeability changes and catalyst release. Subsequent oxidation of —SH groups of vital proteins would cause a loss of function of vital metabolic enzymes or essential —SH proteins important in cell division.[27] Lipid peroxide effects may be localized at specific sites in cells and oxidation of —SH groups of a small fraction of vital —SH proteins essential to metabolism or cell division could cause serious metabolic disturbances, interference with cell division, and lead to the ultimate death of the cell.

Acknowledgements—The authors wish to express their thanks to Professor A. Wormall for advice and interest in this work and to the British Empire Cancer Campaign for a grant to St. Bartholomew's Hospital which helped to support this work.

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