Biochem. J. (1984) **220**, 685–692
Printed in Great Britain

Oxidative interactions between haemoglobin and membrane lipid

A liposome model

Jànos SZEBENI,* Christine C. WINTERBOURN†‡ and Robin W. CARRELL†
*National Institute of Haematology and Blood Transfusion, Budapest, Hungary, and †Pathology Department, Clinical School of Medicine, Christchurch Hospital, Christchurch, New Zealand

(*Received 23 November 1983/Accepted 1 March 1984*)

The relationship between haemoglobin and membrane oxidation was studied using liposomes containing haemoglobin (haemosomes) as a red cell model. Rapid oxidation occurred in haemosomes formed from purified haemoglobin and unsaturated lipid (egg phosphatidylcholines). After 3 h at 37°C most of the haemoglobin was oxidized, predominantly to methaemoglobin with some haemichrome formation. The oxidation of haemoglobin was paralleled by membrane lipid peroxidation as measured by thiobarbituric acid reactivity. These changes were largely abolished by using freshly prepared haemolysate instead of purified haemoglobin, or when haemosomes were prepared with saturated phosphatidylcholines. In haemosomes consisting of fresh haemolysate and saturated phosphatidylcholine, the rate of haemoglobin oxidation at 37°C corresponded to that of non-encapsulated haemolysate, and after 4 months storage at 4°C 45% of oxyhaemoglobin was oxidized. In haemosomes prepared from purified haemoglobin and egg lecithin, α-tocopherol, catalase and ascorbate each protected against both haemoglobin oxidation and lipid peroxidation. Superoxide dismutase or reduced glutathione had no effect. In unsaturated-lipid haemosomes containing haemolysate, the rate of haemoglobin oxidation increased when catalase was inhibited or reduced glutathione was depleted, but after long term incubation only concurrent catalase-inhibition and glutathione depletion could increase thiobarbituric acid reactivity. These results demonstrate a close interdependence between haemoglobin oxidation and lipid peroxidation, and show that constituents of haemolysate strongly protect against both processes. H_2O_2 appears to be an important mediator, with its removal by either catalase or the glutathione/glutathione peroxidase system protecting against both oxidative changes.

Liposomes containing lysate from packed red cells have potential use as a non-immunogenic blood substitute (Djordjevich & Miller, 1980; Gaber *et al.*, 1983; Szebeni *et al.*, 1984). They have been shown to bind O_2 reversibly (Djordjevich & Miller, 1980), but the encapsulated haemoglobin is prone to oxidation and denaturation, depending on the method of haemosome preparation and on their constitution (Gaber *et al.*, 1983; Szebeni *et al.*, 1984). A greater understanding of these changes is required before such a use for haemosomes could be contemplated.

Abbreviations used: GSH, reduced glutathione; BCNU, bis(chloroethyl)nitrosourea.

‡ To whom correspondence should be addressed.

These structures also provide a useful red cell model for studying the inter-relationship between haemoglobin and membrane lipid oxidation and the role of various red cell constituents in protecting against these changes. The oxidative interactions between haemoglobin and the cell membrane is thought to be an important factor in red cell sensescence and in various haemolytic disorders (Babior, 1981; Chiu *et al.*, 1982).

We have studied the interdependence of haemoglobin oxidation and lipid peroxidation in haemosomes where the haemoglobin and lipid organization resembles that of the red cell, and compared the changes that occur when purified haemoglobin or red cell haemolysate is incorporated into the liposomes. We have investigated which oxidative

species are involved in the process and which red cell constituents protect against these changes.

Liposomes were prepared from different phosphatidylcholines and cholesterol, by the hand shaking method, which produces large multilamellar structures (Bangham *et al.*, 1974; Szebeni *et al.*, 1984). Haemoglobin oxidation was determined by measuring the spectra of the haemosomes, and production of thiobarbituric acid-reactive products measured as an index of lipid peroxidation.

Methods

Concentrated human haemolysate ($30 \pm 2\%$) was prepared by lysis and membrane extraction of washed red cells by addition of an equal volume of tetrachloroethylene (Khachaturyan *et al.*, 1979). This extraction was repeated twice and the final dialysis was omitted. Haemoglobin concentrations were determined with Drabkins solution (Riggs, 1981). Purified haemoglobin was prepared by ion-exchange chromatography on DEAE-Sephadex A-50 (Huisman & Dozy, 1965). The haemoglobin A fraction was pooled and concentrated to $30 \pm 2\%$ by ultrafiltration using an Amicon UM 10 membrane. Both chromatography and concentration were carried out at 4°C.

Egg yolk lecithin was either prepared according to Bergelson (1980) and stored at −20°C in benzene, under N_2, or was purchased from Sigma (type IX-E). T.l.c. on silica gel H plates showed both preparations to be devoid of lysolecithin or other contaminants. Haemosomes were prepared by the hand shaking method as follows: 25 μmol of phospholipid and equimolar cholesterol were dissolved in chloroform and evaporated to dryness in a rotary evaporator. Either freshly prepared haemolysate or purified haemoglobin (1.5 ml) and a few glass beads were then added to the thin lipid film and the round-bottom flasks were vigorously machine-shaken for 10 min at room temperature. The haemosomes formed were separated from free haemoglobin by sequential centrifugation in 0.15 M-NaCl/5 mM-Tris/HCl, pH 7.4 (Tris-buffered saline), twice for 20 min at 3000 *g*, 4°C. After the second wash the haemosome sediment was suspended in 10 ml of Tris-buffered saline and incubated at 37°C while vigorously shaking the flasks to prevent sedimentation. At indicated intervals 2.5 ml samples were analysed for haemoglobin oxidation and lipid decomposition.

D-α-Tocopherol, when added, was incorporated in the lipid membrane by dissolving 1 mg (1.5 i.u.) in the chloroform solution of the lipids. This corresponded to an α-tocopherol/egg lecithin molar ratio of about 1:9, or approx. equimolar α-tocopherol and arachidonic acid concentrations. Catalase (1.3 mg/ml), superoxide dismutase (0.1 mg/ml), GSH (5 mM), and ascorbic acid (5 mM) were added to the purified haemoglobin, and BCNU (0.1 mg/ml with 0.5% ethanol), diamide (0.8 mM) and NaN_3 (5 mM) to the haemolysate, where indicated. After addition of these reagents the haemolysate was stood for 10 min at room temperature before haemosome preparation.

The rate of haemoglobin oxidation was determined from the visible spectra of the haemosomes (3–5 mg of lipid/ml) over the range of 500–700 nm, recorded with a Beckman Acta C-III spectrophotometer. The significant light-scattering of haemosomes was compensated for by a liposome blank containing Tris-buffered saline, having the same lipid composition and concentration. The percentages of oxyhaemoglobin (oxyHb), methaemoglobin (metHb) and haemichrome (ferric haemoglobin in which the sixth co-ordination position of the iron is bound to a nitrogen-containing ligand) in the haemosomes were determined from the millimolar extinction coefficients of the tetrameric species (Riggs, 1981; Winterbourn *et al.*, 1976) and the absorbances at the following wavelengths (pH 7.4):

$$A_{577} = 60.0\ [\text{oxyHb}] + 17.8\ [\text{metHb}] + 27.2\ [\text{haemichrome}]$$
$$A_{630} = 0.68\ [\text{oxyHb}] + 14.5\ [\text{metHb}] + 3.7\ [\text{haemichrome}]$$
$$A_{560} = 34.4\ [\text{oxyHb}] + 17.2\ [\text{metHb}] + 34.4\ [\text{haemichrome}]$$

This rearranges to:

$$[\text{oxyHb}] = 29.8\ A_{577} - 9.8\ A_{630} - 22.2\ A_{560}$$
$$[\text{metHb}] = 7.0\ A_{577} + 76.8\ A_{630} - 13.8\ A_{560}$$
$$[\text{Haemichrome}] = -33.2\ A_{577} - 36.0\ A_{630} + 58.2\ A_{560}$$

The baseline absorbance at 700 nm was subtracted from the absorbances at the above wavelengths.

Peroxidative lipid decomposition was estimated by measuring the rate of formation of reactive products as follows (Stocks & Dormandy, 1971). Haemosomes in the 2.5 ml samples were precipitated by 1 ml of 28% (w/v) trichloroacetic acid and 0.1 M-sodium meta-arsenite solution. After sedimenting the precipitate, 3 ml of supernatant and

1ml of 1% thiobarbituric acid in 50mM-NaOH were mixed and heated at 100°C for 15min. Absorbances at 532nm were related to the amount of egg lecithin (in μmol) present. (Each 2.5ml sample initially contained 6.25μmol of egg lecithin, and the phospholipid content of haemosomes was found not to decrease significantly upon washing.) All biochemicals were obtained from Sigma except for superoxide dismutase (Diagnostic Reagents, Thame, Oxon., U.K.); BCNU (Bristol Laboratories, Syracuse, NY, U.S.A.); and L-ascorbic acid (Fisons, Loughborough, Leics., U.K.).

Results

Morphological studies

Coulter counter measurement of egg lecithin/cholesterol liposomes containing haemolysate indicated a broad size range with a mean corpuscular volume of 82 μm^3. Shaking for 1 h instead of 10min during preparation gave a slightly lower size range (mean corpuscular volume 72 μm^3). If Sudan Black was dissolved in the chloroform solution of the lipids before evaporation, the haemosomes were clearly visible by light microscopy, ranging in size between platelets and white cells.

Haemoglobin oxidation in unsaturated liposomes

Encapsulation of haemoglobin in egg lecithin/cholesterol (unsaturated) liposomes results in increased oxidation of the protein (Fig. 1). Purified haemoglobin in free solution autoxidized more rapidly than haemolysate, but the increase in rate of oxidation within the liposomes was considerably greater for purified haemoglobin. The spectra of the purified haemoglobin-containing liposomes show the conversion of oxyhaemoglobin mainly to methaemoglobin although some denaturation to haemichrome was also apparent (Table 1); continued incubation led to the loss of all distinguishable spectral bands (Fig. 2*a*), suggesting total denaturation and possibly dissociation of the haems from the globin. With liposomes containing haemolysate, the spectral changes were much less over the same time period (Fig. 2*b*) and the product was predominantly methaemoglobin with less haemichrome formation (Table 1). On storage at 4°C, most of the haemoglobin in haemolysate-containing unsaturated liposomes was oxidized in 3 weeks, and after 4 months it was almost totally denatured (Fig. 3).

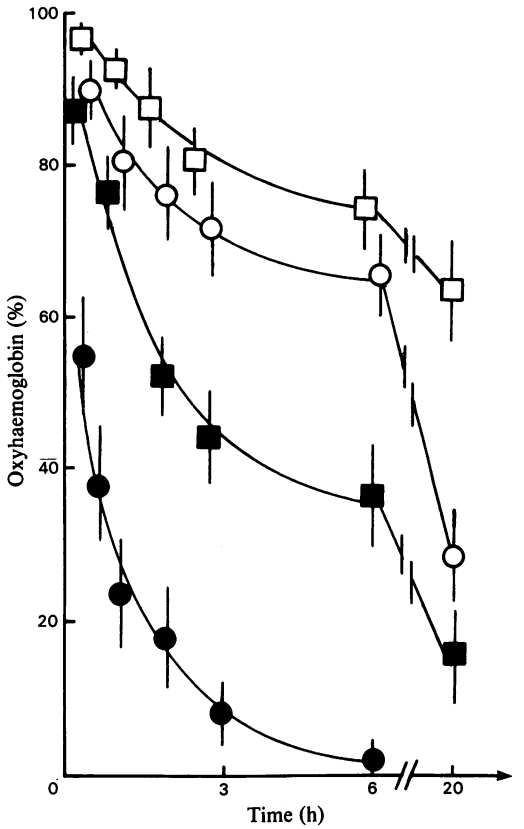

Fig. 1. *Time course of haemoglobin oxidation in unsaturated liposomes compared with in free solution, on incubation at 37°C*

The concentration of the haemoglobin encapsulated into liposomes or in solution was 30g/100ml. The purified haemoglobin was in 50mM-Tris/HCl, pH7.4, but dialysis overnight at 4°C against Tris-buffered saline before haemosome preparation did not affect the oxidation rate. Haemosomes were incubated with shaking in Tris-buffered saline. Haemoglobin solutions were diluted 1:100 before recording spectra. The percentage of oxyhaemoglobin in each solution was calculated from the spectrum as described in the Methods section. The remainder of the haemoglobin was present as methaemoglobin, plus (with haemosomes) a little haemichrome as in Table 1. Means ± S.D. for three to ten experiments are presented. □, Haemolysate; ■, haemolysate-containing liposomes; ○, purified haemoglobin; ●, purified haemoglobin-containing liposomes.

Haemoglobin oxidation in saturated liposomes

The rate of haemoglobin oxidation in liposomes prepared from saturated phospholipid (either dimyristoyl-, distearoyl- or dipalmitoylphosphatidylcholine) and cholesterol was very much slower than in unsaturated liposomes. The rate of oxidation at 37°C of either purified haemoglobin or haemolysate in these liposomes was the same as the rate for each in free solution (shown in Fig. 1). Only methaemoglobin was detected as a reaction product. Oxidation on storage at 4°C was very slow (Fig. 3) and after 4 months 53 ± 3% ($n = 3$) of the haemoglobin remained as oxyhaemoglobin.

Effects of additives on haemoglobin oxidation in haemosomes

When α-tocopherol was added to the liposomal lipid or catalase to the haemoglobin solution, the rate of oxidation of purified haemoglobin was substantially decreased (Fig. 4*a*). Added ascorbate was initially inhibitory, but by 6h this effect was lost (Fig. 4*a*). Superoxide dismutase or GSH had no effect, and catalase was equally protective in the presence or absence of superoxide dismutase.

To attempt to assess whether H_2O_2 or peroxide removal contributed to the protection against haemoglobin oxidation afforded by whole haemolysate, the effects of inhibiting catalase with azide and of oxidizing GSH with diamide (Kosower *et al.*, 1969) plus inhibiting glutathione reductase by BCNU (Frischer & Ahmed, 1977) were examined. BCNU plus diamide caused a slight, and azide a more pronounced, increase in rate of haemoglobin oxidation (Fig. 4*b*). There was no additive effect.

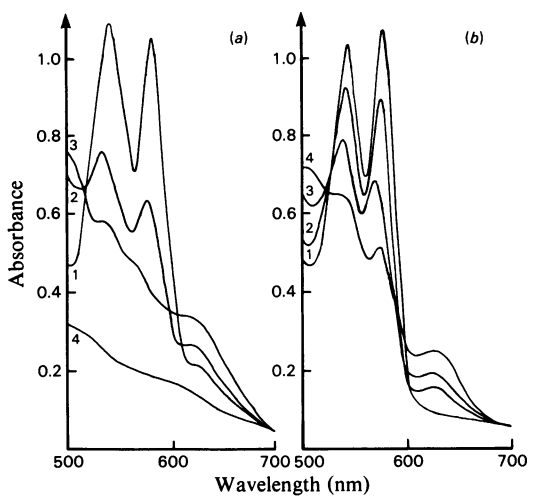

Fig. 2. *Spectral changes of purified haemoglobin (a) and haemolysate (b) encapsulated in unsaturated liposomes*
Typical spectra (from three to six experiments) recorded immediately after preparation (curve 1) and after 3h (2), 6h (3) and 13h (4) incubation at 37°C are shown. Reaction conditions are as for Fig. 1.

Lipid peroxidation in unsaturated haemosomes

Concurrently with spectral measurements of haemoglobin oxidation during incubation at 37°C, formation of thiobarbituric acid-reactive products was measured as an indication of lipid peroxidation in haemosomes. As shown in Fig. 5(*a*), lipid peroxidation occurred very rapidly in the presence of purified haemoglobin whereas it remained at the control (Tris-buffered saline liposome) level for

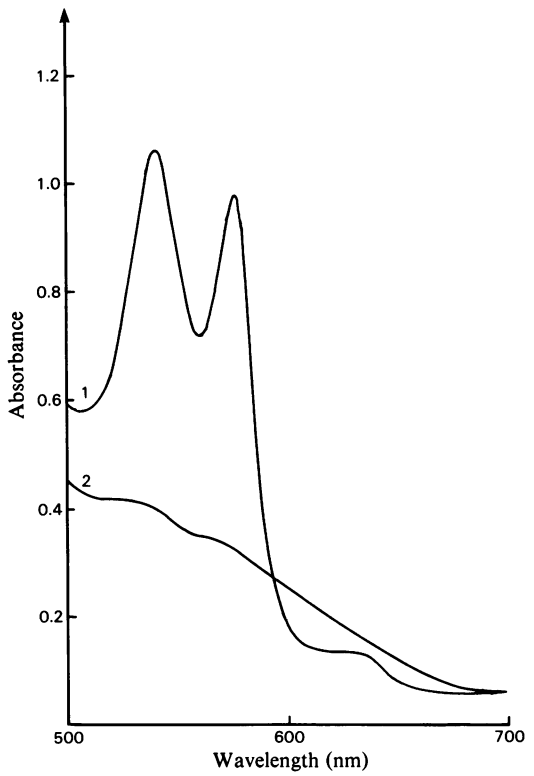

Fig. 3. *Visible spectra of haemoglobin encapsulated as haemolysate in (1) saturated (dipalmitoylphosphatidylcholine/cholesterol), and (2) unsaturated liposomes after 4 months storage at 4°C*
Haemosomes (50μmol of lipid) were stored sedimented in 10ml of Tris-buffered saline. Identical spectra to (1) were obtained with distearoylphosphatidylcholine and dimyristoylphosphatidylcholine liposomes.

Table 1. *Distribution of haemoglobin derivatives in unsaturated haemosomes after 6 h incubation at 37°C*
Means ± S.D. are given for five separate haemosome preparations (purified haemoglobin) or four preparations (haemolysate). Methods of preparation and spectral analysis are given in the Methods section.

	Distribution (%)		
Liposome contents	Oxyhaemoglobin	Methaemoglobin	Haemichrome
Purified haemoglobin	2±3	78±5	20±2
Haemolysate	39±8	49±6	10±6

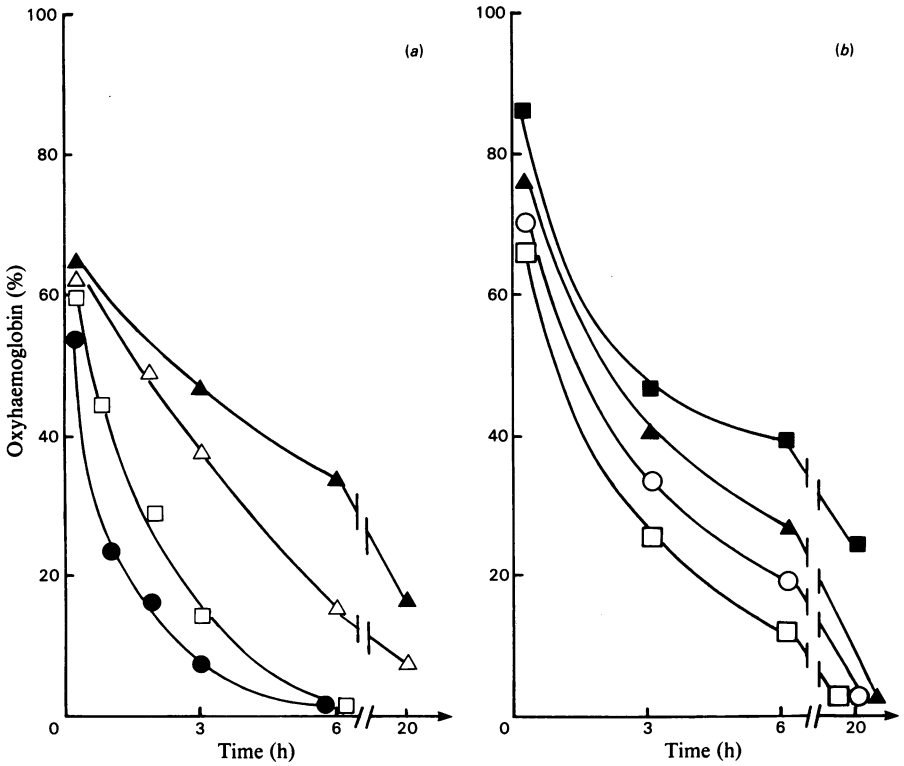

Fig. 4. *Rates of oxidation of purified haemoglobin (a) and undiluted haemolysate (b) in unsaturated liposomes* (*a*), ●, No additives; ▲, added α-tocopherol; △, catalase; □, ascorbate. (*b*) ■, No additives; □, added NaN_3; ▲, BCNU and diamide; ○, NaN_3 + BCNU and diamide. The points represent typical values from at least three similar experiments. Methodological details and reagent concentrations are described in the Methods section and in the legend to Fig. 1.

haemolysate-containing liposomes. In general there was good correlation between the extent of lipid peroxidation and haemoglobin oxidation, although it was noted that lipid peroxidation was less when the purified haemoglobin initially contained high concentrations of methaemoglobin.

Paralleling their effects on haemoglobin oxidation, α-tocopherol and catalase added to liposomes containing purified haemoglobin each completely inhibited lipid peroxidation, and ascorbate gave short term protection (Fig. 5*b*). Superoxide dismutase and GSH were without effect. With haemolysate-containing liposomes, azide or BCNU plus diamide added separately did not result in increased thiobarbituric acid reactivity (Fig. 5*c*). However, when all these reagents were added together, lipid peroxidation was significantly increased but only after longer than 6h incubation (Fig. 5*c*).

Discussion

In this study we have prepared phospholipid/cholesterol liposomes containing haemoglobin at physiological concentrations (haemosomes), and studied oxidative interactions between the components. We have used this system both as a model to help understand these interactions in the erythrocyte, and also because if haemosomes are to be useful as a blood substitute, oxidative destruction must be minimized. A major proportion of the haemosomes were shown by Coulter counter measurement and the stained films to be in the blood cell size range. However, the hand shaking technique produces multilamellar structures, and they obviously differ from erythrocytes in having no protein cytoskeleton or negatively charged inner membrane surface.

It is known that haemoglobin and other haem proteins can catalyse the peroxidation of polyunsaturated fatty acids (Tappel, 1955; Tappel *et al.*, 1961; Nakamura & Nishida, 1971; Haurowitz *et al.*, 1973; Ursini *et al.*, 1981), and that haemoglobin is oxidized by unsaturated fatty acids and O_2 (Haurowitz *et al.*, 1941) or by peroxides, e.g. *t*-butylhydroperoxide (Trotta *et al.*, 1982, 1983). High concentrations of haemoglobin can be inhibi-

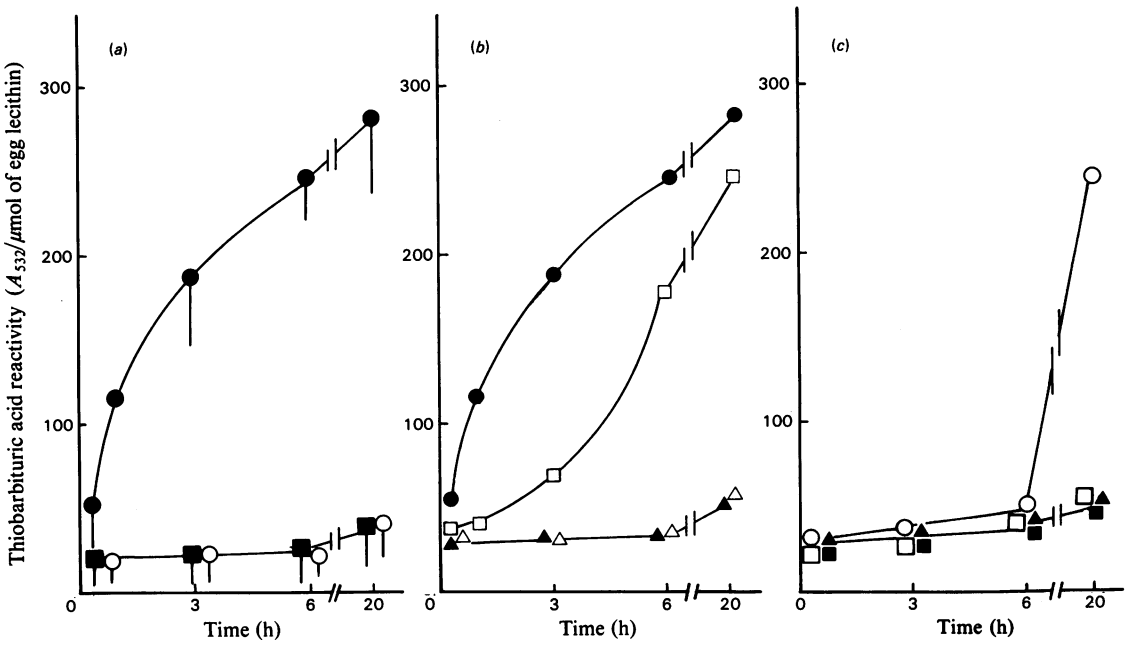

Fig. 5. *Measurement of thiobarbituric acid reactivity in haemosomes incubated at 37°C*
(*a*) Unsaturated liposomes containing either purified haemoglobin (●), whole haemolysate (■) or Tris-buffered saline (○). Means ± S.D. of three to ten experiments are presented. (*b*) The effects of catalase (△), α-tocopherol (▲), and ascorbate (□) in unsaturated liposomes containing purified haemoglobin; (●), no additives, transposed from (*a*). (*c*) The effects of NaN_3 (□), BCNU and diamide (▲), and NaN_3 + BCNU and diamide (○) in unsaturated liposomes containing haemolysate; (■), no additives, transposed from (*a*). Results from a typical experiment are shown. Methodological details and reagent concentrations are given in the Methods section and in the legend to Fig. 1.

tory (Lewis & Wills, 1963; Kendrick & Watts, 1969; Nakamura & Nishida, 1971), however, and the situation in the red cell is less clear. Enclosure of haemoglobin in liposomes would be expected to protect against peroxidation, since the protein should be separated from the polyunsaturated fatty acids in the hydrophobic core of the bilayer by the hydrophilic lipid head groups, and in the absence of phosphatidylserine no binding to lipid monolayers should occur (Szundi *et al.*, 1980). Nevertheless, we measured rapid oxidation of purified haemoglobin encapsulated in liposomes, with concurrent increase in thiobarbituric acid-reactivity, a generally accepted measure of lipid peroxidation. The primary haemoglobin oxidation product was methaemoglobin, with some haemichrome and possibly other denaturated products becoming evident after longer incubation.

Oxidation of both haemolysate and purified haemoglobin occurred much more rapidly in liposomes prepared from unsaturated lipid than in solution but enclosure in saturated liposomes made little difference to the haemoglobin oxidation rate. In unsaturated liposomes containing purified haemoglobin, lipid peroxidation occurred in parallel with the oxidation of haemoglobin, suggesting a close link between the two processes. The observed inhibition of haemoglobin oxidation as well as lipid peroxidation by α-tocopherol is further support for this proposal. Interdependence between these processes was not absolute, however, because there was no detectable lipid peroxidation with haemolysate over the period studied in spite of substantial haemoglobin oxidation. Another factor that affected the amount of lipid peroxidation was the level of methaemoglobin in the encapsulated solution. No peroxidation was detected when purified methaemoglobin was used, and oxidation of oxyhaemoglobin was accompanied by relatively less lipid peroxidation when the initial ratio of methaemoglobin to oxyhaemoglobin was increased. This suggests that oxyhaemoglobin is required for lipid peroxidation, and a protective effect of methaemoglobin, similar to that observed by Trotta *et al.* (1982, 1983) with *t*-butylhydroperoxide in erythrocytes.

Rates of haemoglobin oxidation and lipid peroxidation were much higher in liposomes containing purified haemoglobin compared with haemolysate, implying that some haemolysate constituents were strongly protective. Two approaches were made to investigating this effect. Various

haemolysate components were added back into purified haemoglobin, and peroxide-metabolizing enzymes in haemolysate were inhibited. Catalase, which breaks down H_2O_2, was inhibited by NaN_3, and the glutathione pathway, which can metabolize either H_2O_2 or lipid peroxides (Wendel, 1981), was inhibited by adding diamide to oxidize GSH (Kosower *et al.*, 1969), and BCNU to inhibit glutathione reductase (Frischer & Ahmed, 1977). However, we cannot be certain that inhibition of these enzymes is the only effect of these agents in the haemosome system, and the results must be interpreted with this qualification. With purified haemoglobin, inhibition of both haemoglobin oxidation and lipid peroxidation by catalase, but not by superoxide dismutase, implies a major mediating role for H_2O_2 but not superoxide. Ascorbate was protective over shorter time periods, presumably until it had all been oxidized. Its effect may have been due to its reducing or scavenging haemoglobin or radical intermediates. No similar effect of GSH was apparent. Ascorbate but not GSH has also been found to inhibit H_2O_2-induced red cell lipid peroxidation (Stocks & Dormandy, 1971). The close parallelism between the time course of inhibition of haemoglobin oxidation and lipid peroxidation by ascorbate in our studies provides further evidence for these two processes being interdependent. The effect of the inhibitors of peroxide metabolism on oxidation in haemolysate-containing liposomes are also compatible with H_2O_2 playing a key role in the process. Although either inhibitory regime increased the rate of haemoglobin oxidation, both were required for lipid peroxidation to occur. It is often postulated that GSH and glutathione peroxidase provide the main protection against H_2O_2 in the red cell (Nicholls, 1972; Chiu *et al.*, 1982) but the findings in this haemosome model support other evidence (Aebi & Suter, 1974) that the glutathione system and catalase provide mutual backup in this role.

In summary, we have found that rapid haemoglobin oxidation and lipid peroxidation occur in unsaturated liposomes, and are inhibited by α-tocopherol and some components of the haemolysate. Some of our findings parallel those in the red cell and haemosomes appear to be useful for probing the mechanism of these processes. Our results suggest an oxidative chain, with lipid peroxidation products oxidizing haemoglobin, and products of this reaction in turn causing more peroxidation. Although the overall mechanism probably involves more than one reaction sequence, we have demonstrated that H_2O_2 is an important intermediate. The process could be started by haemoglobin oxidation, which produces H_2O_2 (Winterbourn *et al.*, 1976) or alternatively, via the reaction of pre-existing lipid peroxides (invariably present in lipid preparations) with oxyhaemoglobin. This reaction produces lipid alkoxy radicals and methaemoglobin (Thornalley *et al.*, 1983) so H_2O_2 would have to be formed in a subsequent step. Another alternative is hydrogen abstraction from polyunsaturated fatty acids (LH) by oxyhaemoglobin (HbO_2):

$$HbO_2 + LH + H^+ \rightarrow H_2O_2 + L^\cdot + Hb^{3+}$$

Further studies are needed to elucidate this mechanism.

For use as a blood substitute, liposomes containing purified haemoglobin are obviously unsatisfactory unless reagents protective against oxidation are added. Haemolysate gives much less oxidation, and haemosomes containing haemolysate and prepared from saturated phospholipids show the greatest stability. This is in agreement with other recent findings (Gaber *et al.*, 1983; Szebeni *et al.*, 1984). Although unilamellar liposomes would have the advantage of a higher haemoglobin-carrying capacity, preparative methods such as ether injection result in gross denaturation of the encapsulated haemoglobin (Szebeni *et al.*, 1984) and at this stage they are not a viable alternative to the multilamellar vesicles used in this study. With avoidance of organic solvents, saturated liposomes containing whole haemolysate appear to show most promise for further investigation in this area.

This work was supported by the Medical Research Council of New Zealand. Sincere thanks are due to Margaret Vissers and David Williamson for their help in this work. The technical assistance of Felicity White is gratefully acknowledged. J. S. held a visiting fellowship at the Christchurch Clinical School of Medicine.

References

Aebi, H. & Suter, H. (1974) in *Glutathione* (Flohé, L., Benöhr, H. C., Sies, H., Waller, H. D. & Wendel, A., eds.), p. 192, Thieme, Stuttgart

Babior, B. M. (1981) in *The Function of Red Blood Cells: Erythrocyte Pathobiology* (Wallach, D. F. H., ed.), pp. 173–195, Alan R. Liss, New York

Bangham, A. D., Hill, M. W. & Miller, N. G. (1974) *Methods Membr. Biol.* **1**, 1–68

Bergelson, L. D. (1980) *Lipid Biochemical Preparations*, pp. 128–129, Elsevier/North-Holland Biomedical Press, Amsterdam, New York and Oxford

Chiu, D., Lubin, B. & Shohet, S. B. (1982) in *Free Radicals in Biology* (Pryor, W. A., ed.), vol. 5, pp. 115–160, Academic Press, New York, San Francisco and London

Djordjevich, L. & Miller, I. F. (1980) *Exp. Hematol.* **8**, 584–592

Frischer, H. & Ahmed, T. (1977) *J. Lab. Clin. Med.* **89**, 1080–1091
Gaber, B. P., Yager, P., Sheridan, J. P. & Chang, E. L. (1983) *FEBS Lett.* **153**, 285–288
Haurowitz, F., Groh, M. & Gansinger, G. (1973) *J. Biol. Chem.* **248**, 3810–3818
Haurowitz, P., Schwerin, P. & Yenson, M. M. (1941) *J. Biol. Chem.* **140**, 353–359
Huisman, T. H. J. & Dozy, A. M. (1965) *J. Chromatogr.* **19**, 160–169
Kendrick, J. & Watts, B. M. (1969) *Lipids* **4**, 454–458
Khachaturyan, A. A., Vyazova, E. P., Morozova, G. M. & Rozenberg, G. Ya. (1979) *Probl. Hematol.* **24**, 58–60
Kosower, N. S., Kosower, E. M., Wertheim, B. & Cerrea, W. S. (1969) *Biochem. Biophys. Res. Commun.* **37**, 593–596
Lewis, S. E. & Wills, E. D. (1963) *Biochim. Biophys. Acta* **70**, 336–338
Nakamura, Y. & Nishida, T. (1971) *J. Lipid Res.* **12**, 149–154
Nichols, P. (1972) *Biochim. Biophys. Acta* **279**, 306–309
Riggs, A. (1981) *Methods Enzymol.* **76**, 5–29
Stocks, J. & Dormandy, T. L. (1971) *Br. J. Haematol.* **20**, 95–111
Szebeni, J., Breuer, J. H., Szelényi, J. G., Báthori, G., Lelkes, G. & Hollán, S. R. (1984) *Biochim. Biophys. Acta*, **798**, 60–67
Szundi, I., Szelényi, J. G., Breuer, J. H. & Bérczi, A. (1980) *Biochim. Biophys. Acta* **595**, 41–46
Tappel, A. L. (1955) *J. Biol. Chem.* **217**, 721–733
Tappel, A. L., Brown, W. D., Zalkin, H. & Maier, V. P. (1961) *J. Am. Oil Chem. Soc.* **38**, 5–9
Thornalley, P. J., Trotta, R. J. & Stern, A. (1983) *Biochim. Biophys. Acta* **759**, 16–22
Trotta, R. J., Sullivan, S. G. & Stern, A. (1982) *Biochem. J.* **204**, 405–415
Trotta, R. J., Sullivan, S. G. & Stern, A. (1983) *Biochem. J.* **212**, 759–772
Ursini, F., Maiorino, M., Ferri, L., Valente, M. & Gregolin, C. (1981) *J. Inorg. Biochem.* **15**, 163–169
Wendel, A. (1981) *Methods Enzymol.* **77**, 325–333
Winterbourn, C. C., McGrath, B. M. & Carrell, R. W. (1976) *Biochem. J.* **155**, 493–502