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# Studies on hemolysis of human erythrocytes by linoleic acid

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**Abstract.** The results presented in this paper show that lysis of human erythrocytes by linoleic acid is not caused by peroxidation of the fatty acid. Peroxidase, superoxide dismutase and scavengers of $O_2^{\bar{}}\cdot$ and OH had no effect on the lysis while catalase showed only marginal inhibition suggesting that $O_2^{\bar{}}$, OH , $O_2^{\bar{}}$ and $H_2O_2$ do not play any direct role in hemolysis by linoleic acid. Generators of $H_2O_2$ inhibited the lysis completely and methemoglobin cells were more resistant to hemolysis by linoleic acid. The fatty acid did neither bind to nor fomed complex with red cell ghosts. Membrane oxidation of sulphydryl groups was also not involved in the lysis. *β*-Carotene, retinol and bile salts enhanced the lysis, while, cholesterol but not cholesterol acetate, inhibited it. Taurocholate-pretreated cells were more susceptible to linoleic acid lysis. These observations suggested-that lysis by linoleic acid may be due to its detergent property.

**Keywords.** Hemolysis; linoleic acid; superoxide anion; hydroxyl radical; taurocholate; detergent action.

## Introduction

In an earlier report, we demonstrated that linoleic acid-induced lysis of normal erythrocytes *in vitro* is independent of the peroxidation observed at higher concentrations of the acid and was related to the changes in the cell permeability (Bai *et al.,* 1979). Retinol-induced lysis of erythrocytes *in vitro* was elegantly explained as being primarily due to its detergent-like action (Lucy and Dingle, 1964; Lucy, 1970). Recent reports (Weltzein *et al.,* 1977; Billington *et al.,* 1977; Segal and Goldzweig, 1978) provide considerable evidence for mechanism for lysis by detergent action of lysolecithin, taurocholate and several other detergents. On the other hand, involvement of reactive radicals, $O_2^{\bar{}}\cdot$ and $OH_2$ and $H_2O_2$ in lysis of human cells and linoleate oxidation was demonstrated (Kellogg and Fridovich, 1975; Kellogg and Fridovich, 1977). Although fatty acids such as palmitic, stearic, myristic and caproic acids were not hemolytic (Bai *et al.,* 1979), it was of interest to investigate the mechanism of lysis induced by linoleic acid, which has a detergent-like action and is a peroxidisable substrate.

## Materials and methods

### *Chemicals*

Linoleic acid (pure), diethyl dithiocarbamate, and silica gel were purchased from British Drug House, Poole, England. Menadione, phenyl hydrazine 1,2-naphtha-quinone-4-sulphonic acid, lecithin, cholesterol, cholesterol acetate, bovine serum albumin, sodium benzoate, L-histidine, glucose, mannitol, 5,5'-dithiobis-2-nitro benzoic acid and dimethyl sulphoxide were products of Sigma Chemical Company, St. Louis, MO, U.S.A. $\beta$-Carotene and hydroquinone were from E-Merck, AG-Darmastadt, Germany. Sodium taurocholate was the product of Koch-Light Laboratories, Colnbrook, Bucks, England. Retinol was from Hoffman-La-Roche, Basle, Switzerland. Horse radish peroxidase (350-500 units/mg) and catalase (2,800 units/mg) were from SISCO Research Laboratories, Bombay. Superoxide dismutase was prepared from bovine erythrocytes according to the procedure of McCord and Fridovich (1969) except that the last step involving DE-32 chromatography was omitted. The preparation had a specific activity of 800 units/mg when assayed by the diansidine method . Linoleic acid hydroperoxide was prepared by the method of O'Brien (1969).

The preparation of erythrocytes, measurement of hemolysis and lipid peroxi dation and incorporation of additives into the incubation system were as described earlier (Bai *et al.,* 1979). Phospholipid liposomes were prepared from fresh ox-brain (100 g) by the method of Guttridge (1977).

The ghosts were prepared as described by Hanahan and Ekhlom (1974). The washed, packed white-ghosts were finally suspended in saline-phosphate buffer pH 7·4 to a volume equal to that of the original packed cells.

*Linoleic acid binding :* Two ml of the ghost suspension (500$\mu$g protein) was mixed with 2 mg of linoleic acid dissolved in 0·1 ml ethanol and incubated at 37° C for 60 min. The ghosts were sedimented by centrifugation at 20,000 g for 40 min at 4° C, washed thrice with saline-phosphate buffer, pH 7·4, and resuspended in 1 ml of 10 mM phosphate buffer, pH 7·4, and mixed with an equal volume of 1% (v/v) Triton X-100. The bound linoleic acid was determined by the method of Dole (1956) as modified by Trout *et al.* (1960). Ghosts incubated without linoleic acid served as control.

*Oxidation of sulphydryl groups of the membranes* : Red cell ghosts were incubated with linoleic acid as described above. Oxidation of SH group was measured by the method of Szeinberg and Clegan (1964). Ghosts incubated alone served as control.

*Thin layer chromatography* : The assay system containing 0·25 mg of linoleic acid in 0 05 ml of ethanol and 0 2 ml of packed cells or equivalent cell-ghosts in a final volume of 6 ml was incubated at 37° C for 60 min. Controls omitting linoleic acid were also incubated. At the end of incubation, 2ml of chloroform was added to extract the lipid components. The chloroform extract was used for thin layer chromatographic (TLC) analysis on silica gel. The TLC plates were developed with petroleum ether/benzene/acetone (1: 1: 1, v/v/v). The method of Stahl (1958) for organic peroxides were modified for locating linoleate peroxides

as described by Kellogg and Fridovich (1975). Thus, 1·0 ml of 4% KI was mixed with 4·0 ml of acetic acid, and this mixture was immediately sprayed, followed after 6 min by a spray of fresh 1% solution of starch. Areas of the chromatogram containing peroxides were stained purple.

*Pretreatment of red cell ghosts with sodium taurocholate* : One ml of packed cells were treated with sodium taurocholate, (final concentration 0·l%,w/v) in saline phosphate pH 7.4, in a total volume of 5 ml, and incubated at 37° C for 10 min, chilled immediately and the cells sedimented, washed thrice with saline-phosphate buffer, pH 7·4 and used for studying hemolysis by inoleic acid.

## Results and discussion

Table 1 summarises the effect of $H_2O_2$ and compounds known to generate $H_2O_2$ on hemolysis by linoleic acid. All the compounds tested showed a pronounced inhibition of the lysis. Cchen and Hcchstein (1965) have reported that $H_2O_2$ was responsible for lysis of red cells exposed to phenylhydrazine, primaquine and aniline. They also showed that phenylhydrazine, menadione and naphthaquinone when injected into animals produce $H_2O_2$ and *in vivo* hemolysis (Cchen and Hochstein, 1964). It is interesting, as our results show, that the human erythrocytes are pro-

**Table 1.** Effect of $H_2O_2$ and compounds known to generate $H_2O_2$ on the hemolysis of red cells by linoleic acid.

| Additions (nM) | Hemolysis (%) Time (min) | | |
|---|---|---|---|
| | 0 | 30 | 60 |
| None | 0 | 3 | 3 |
| Linoleic acid | 3 | 48 | 100 |
| $H_2O_2$ (440) | 5 | 8 | 10 |
| Linoleic acid + $H_2O_2$ (440) | 0 | 7 | 10 |
| Linoleic acid + menadione (1·5) | 2 | 15 | 28 |
| Linoleic acid + phenyl hydrazine (1·5) | 2 | 8 | 14 |
| Linoleic acid + 1–2, naphthaquinone 4-sulphonic acid (1·5) | 3 | 10 | 16 |
| Linoleic acid + hydroquinone (2·0) | 5 | 13 | 26 |
| Linoleic acid + linoleic acid hydroperoxide | 5 | 8 | 10 |
| Linoleic acid + preincubated liposomes | 4 | 6 | 13 |

The incubation mixture contained 250 $\mu$g of linoleic acid dissolved in 0·05 ml aldehyde-free ethanol to which 5·75 ml saline-phosphate buffer (pH 7·4) was added, mixed into a uniform dispersion, and 0·2 ml packed cells were introduced. Incubation at 37° C for 60 min. The percentage of hemolysis was determined by measuring the absorbance of the supernatant at 520 nm. Additives were added in the buffer. Linoleic acid hydroperoxide and pre-incubated liposomes contained peroxide equivalent to 100 nM of malondialdihyde, as assayed by 2-thiobarbituric acid, test.

tected from linoleic acid lysis by $H_2O_2$ and generators of $H_2O_2$. On the contrary, the lysis of cells from retinol-dosed rats is shown to be enhanced by $H_2O_2$ (Kartha and Krishnamurthy, 1978). For hemolysis of cells.from hypervitaminotic A or $\alpha$-tocopherol-deficient rat, $H_2O_2$, dialuric acid or glutathione act as an oxidant stress factor but only in the case of $\alpha$-tocophero1-deficient cells, such lysis is accompanied by peroxidation of stromal lipids (Kartha and Krishnamurthy 1978; Krishnamurthy and Bieri, 1961) Recent reports, however, suggest that even in the case of cells from $\alpha$-tocopherol deficient rats, the oxidation of membrane lipids and lysis are a concurrent but not a consecutive phenomenon (Brownlee *et al.,* 1977). *In vitro* hemolysis of rat erythrocytes by retinol and the hemolysis of cells from vitamin E deficient rats are initiated by hydroxyl radicals (unpublished observations). Linoleic acid lysis of human cells is not accompanied by stromal membrane peroxidation and is not due to the oxidation of the added fatty acid (Bai *et al.,* 1979) Further, linoleic acid hydroperoxide as well as peroxidised ox-brain liposomes formed by incubation with $Fe^{2+}$ inhibited the fatty acid lysis (table 1).

Superoxide anion generated by xanthine oxidase and acetaldehyde (Kellog and Fridovich, 1977) or by the autoxidation of dihydroxyfumaric acid (Goldberg and Stern, 1977) was shown to lyse human erythrocytes. The possible involvement of $O_2^-$, OH· and $H_2O_2$ in linoleic acid induced hemolysis was tested using superoxide dismutase, catalase and peroxidase (table 2). Peroxidase, catalase and super-

**Table 2.** Effect of superoxide dismutase, catalase, peroxidase and inhibitors of superoxide dismutase and catalase on hemolysis of red cells by linoleic acid.

| Additions | Hemolysis (%) Time (min) 0 | 30 | 60 |
|---|---|---|---|
| None | 0 | 3 | 5 |
| Linoleic acid | 0 | 40 | 96 |
| Peroxidase | 0 | 5 | 6 |
| Linoleic acid + peroxidase | 1 | 45 | 98 |
| Catalase | 0 | 3 | 6 |
| Linoleic acid + catalase | 0 | 30 | 68 |
| Superoxide dismutase | 0 | 3 | 5 |
| Linoleic acid + superoxide dismutase | 0 | 38 | 94 |
| Linoleic acid + superoxide dismutase + catalase | 0 | 32 | 70 |
| Diethyl dithiocarbocarbamate | 0 | 42 | 100 |
| Sodium azide | 0 | 2 | 5 |
| Linoleic acid + sodium azide | 2 | 45 | 100 |

Details are given under table 1. One ml of the incubation medium containing peroxidase (horse radish), 10 units, catalase 2,800 units, or superoxide dismutase 10 units was used. Diethyldithiocarbamate 2·5 mM and sodium azide 0·1 mM were incorporated in the buffer. Incubation at 37°C.

oxide dismutase did not have any marked effect on the lysis by fatty acid although catalase showed a marginal inhibition of 10–15%. Simultaneous presence of superoxide dismutase and catalase had no additional effect on lysis by linoleic acid, even though superoxide dismutase has been shown to reduce the inactivation of catalase (Kellogg and Fridovich, 1977). Peroxidase and catalase decompose $H_2O_2$, while superoxide dismutase dismutases $O_2^{\overline{\cdot}}\cdot$, in erythrocytes. This would indicate that the fatty acid lysis is not initiated by $O_2^{\overline{\cdot}}\cdot$, OH·or $H_2O_2$. Diethyl-dithiocarbamate and sodium azide, specific inhibitors of superoxide dismutase and catalase did not influence the course of hemolysis by linoleic acid indicating again that the lysis by fatty acid proceeded by mechanism not involving reactive radicals.

The results in table 3 further rule out the involvement of OH·radical in the lysis by linoleic acid since glucose, mannitol, sodium benzoate, *t*-butanol and di-

**Table 3.** Effect of scavengers of singlet $O_2$ and hydroxyl radical on erythrocyte hemolysis by linoleic acid.

| Additions (nM) | Hemolysis (%) | | |
|---|---|---|---|
| | Time (min) | | |
| | 0 | 30 | 60 |
| None | 0 | 2 | 5 |
| Linoleic acid | 2 | 40 | 96 |
| Linoleic acid + glucose (10) | 5 | 50 | 94 |
| Linoleic acid + mannitol (10) | 0 | 33 | 66 |
| Linoleic acid + sodium benzoate (1) | 0 | 42 | 98 |
| Linoleic acid + dimethyl sulphoxide (1·3) | 6 | 45 | 100 |
| Linoleic acid + *t*-butanol (100) | 2 | 40 | 92 |
| Linoleic acid + histidine (0·5) | 0 | 18 | 15 |
| Linoleic acid + $\beta$-carotene (0·01) | 0 | 100 | 100 |
| Linoleic acid + xanthine (0·5) | 0 | 32 | 85 |
| Linoleic acid + urate (0·5) | 0 | 28 | 82 |

Details as given under table 1. Lipid-soluble factors included in 0·05 ml ethanol containing linoleic acid; and water-soluble substances were dissolved in the buffer. Incubation at 37°C.

methyl sulphoxide, scavengers of OH·radical did not inhibit the lysis. However, singlet $O_2$ scavengers, L-histidine and $\beta$-carotene showed anomalous behaviour– $\beta$-carotene enormously enhanced the lysis (100% in 30 min compared to 40% by linoleic acid alone), while histidine inhibited it by 85% at 60 min incubation. L-Histidine or $\beta$-carotene by itself did not produce any appreciable hemolysis even after 60 min at 37° C. This result could not therefore be taken as a positive indication for the involvement of singlet $O_2$.

Recovery of linoleic acid after incubating the erythrocytes membrane with linoleic acid showed that the added fatty acid was not destroyed during incubation. Further, it was observed that linoleic acid, was not bound to the membrane com-

ponents (table 4). The suggestions that lysis was not due to peroxidation of linoleic acid or a membrane lipid was confirmed by extracting the lipids after hemolysis by linoleic acid with chloroform and analysing for peroxides. Fatty acid

**Table 4.** Binding of fatty acid to and oxidation of membrane bound-SH groups in red cell ghosts treated with linoleic acid.

| | Contiol | Linoleic acid treated |
|---|---|---|
| Linoleic acid bound[a] | 8 ± 2·5 | 10 ± 3·1 |
| Sulphydryl groups[b] | 3·5 ± 0·8 | 4·1 ± 0·9 |

[a] μg of linoleic acid bound per mg of ghost protein.
[b] Values are no. of SH groups, × $10^{-16}$ mg of ghost-protein.
Protein was estimated by Lowry *et al.* (1951).

peroxides could not be detected on the chromatograms. In another experiment, when red cell-ghosts were incubated with linoleic acid, hydroperoxides were not detected. Hydroperoxides were detected when linoleic acid or artificial liposomes were exposed to acetaldehyde and xanthine oxidase (Kellogg and Fridovich, 1977).

Since the above results indicated that the absence of lipid peroxidation, the membrane-SH groups before and after treatment with linoleic acid was measured. The oxidation of membrane-SH groups could not be detected during lysis by fatty acid (table 4). Hemolysis of erythrocytes by menadione or dihydroxyfumaric acid has been shown to involve membrane-SH oxidation (Mezick *et al.,* 1970; Goldberg and Stem, 1977).

Since fatty acids are known to have detergent property, the lytic action of linoleic acid might be explained by its detergent effect. To investigate this possibility, cells were pretreated with sodium taurocholate (0·1%) at 37° C for 10 min and the washed cells were then exposed to linoleic acid (table 5). Pretreated-cells showed no autchemolysis upto 2h, even though the susceptibility to linoleic acid was remarkably enhanced by taurocholate pretreatment. Complete lysis of the pretreated cells was noticed within 15 min when exposed to the fatty acid. Sodium taurocholate treatment resulted in the loss of phospholipid (10–20%) from sheep (Billington *et al.,* 1977) and human erythrocytes (Coleman and Holdsworth, 1976). Lecithin exhibited a marginal effect, while cholesterol prevented the lysis completely (table 5). Lecithin and cholesterol being the major lipid components of the erythrocyte membrane, the primary effect of linoleic acid might be on these lipid components. This was further confirmed by the observation that cholesterol acetate could not replace cholesterol (the major sterol component of erythrocytes is cholesterol and not its ester). Retinol, another lipid soluble antioxidant (Kartha and Krishnamurthy, 1977) and hemolytic agent (Kartha and Krishnamurthy, 1978) enhanced hemolysis by linoleic acid. The inhibiton of linoleic acid induced hemolysis by albumin could be due to its binding to the fatty acid (table 5).

**Table 5.** Effect of lecithin, cholesterol, retinol, albumin and taurocholate on hemolysis of red cells by linoieic acid.

| Additions | Hemolysis (%) | | | |
|---|---|---|---|---|
| | Time (min) | | | |
| | 0 | 15 | 30 | 60 |
| None | 0 | 2 | 2 | 5 |
| Linoleic acid[a] | 0 | 10 | 38 | 100 |
| Lecithin | 0 | 8 | 10 | 12 |
| Linoleic acid + lecithin | 0 | 6 | 30 | 96 |
| Cholesterol | 0 | 2 | 2 | 3 |
| Linoleic acid + cholesterol | 0 | 3 | 3 | 3 |
| Cholesterol acetate | 2 | 4 | 4 | 6 |
| Linoleic acid + cholesterol acetate | 2 | 8 | 32 | 92 |
| Retinol | 11 | 45 | 56 | 100 |
| Linoleic acid + retinol[b] | 26 | 100 | 100 | 100 |
| Albumin | 0 | 2 | 2 | 5 |
| Linoleic acid + albumin | 0 | 8 | 10 | 40 |
| Taurocholate pre-treated cells[c] | 0 | 5 | 8 | 8 |
| Taurocholate pre-treated cells + linoleic acid | 10 | 100 | 100 | 100 |

[a] Linoleic acid, 250 $\mu$g in 0·05 ml ethanol. [b] Retinol, 35 $\mu$g in 0·05 ml ethanol.
[c] Sodium taurocholate pre-treated cells prepared as detailed in text and used in the place of normal cells. Other details as given under tables 1 and 3. Other additives were added at concentration of mg in a final volume of 6 ml.

The results of this study suggest lysis by linoieic acid was primarily due to its detergent-like action and not due to $O_2^-$ OH·, $O_2$ or $H_2O_2$. Although linoieic acid is a known autoxidisable substrate and the lysis of α-tocopherol-deficient cells has been reported to be due to stromal lipid autoxidation, our results could discount such a mechanism for linoieic acid-induced lysis of human erythrocytes.

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