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# MEMBRANE PROTEIN CHANGES INDUCED BY *tert*-BUTYL HYDROPEROXIDE IN RED BLOOD CELLS

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**Red cells were incubated in the presence of *t*-butyl hydroperoxide and effects on red cell membrane proteins were studied by SDS-polyacrylamide gel electrophoresis. *t*-Butyl hydroperoxide caused diminution in intensity of all major cytoskeletal bands with the concomitant formation of high molecular weight material. Membrane glycoproteins were unaffected. *t*-Butyl hydroperoxide increased hemoglobin binding to ghosts. After dissolution in SDS and β-mercaptoethanol, membrane-bound hemoglobin appeared on the gels in the form of monomers and crosslinked polymers of hemoglobin or globin chains. Crosslinking was partially prevented by metabolism of *t*-butyl hydroperoxide by the hexose monophosphate shunt except in methemoglobin-containing red cells where reaction with methemoglobin accounted for most of the consumption of *t*-butyl hydroperoxide. Metal chelators, deferoxamine mesylate and diethylenetriaminepentaacetic acid, had no effect on membrane protein changes. Butylated hydroxytoluene, diphenylamine and ascorbate, compounds that inhibit *t*-butyl hydroperoxide-induced red cell membrane lipid peroxidation, had no effect on *t*-butyl hydroperoxide-induced membrane protein changes. These results suggest that membrane proteins and membrane lipids have different mechanisms of peroxidant damage.**

## Introduction

Hydroperoxides are implicated in the mechanism of oxidative hemolysis [1]. The organic hydroperoxide, *t*-butyl hydroperoxide has been used as a model in red cells for understanding this process. *t*-Butyl hydroperoxide has been shown to have effects on red cell membrane cytoskeletal proteins [2,3], red cell hydrodynamics [2] and red cell membrane lipids [3–9]. In this report we have incubated red cells in the presence of *t*-butyl hydroperoxide and have evaluated the changes in red cell membrane proteins in relationship to glucose metabolism, ligand state of hemoglobin and the presence of antioxidants and metal chelators.

Abbreviation: DETAPAC, diethylenetriaminepentaacetic acid.



## Materials and Methods

*Red cell preparations.* Adult human blood was drawn daily into a test tube containing a small amount of 3.8 percent sodium citrate solution. After centrifugation, plasma and white cells were removed, and red cells were washed three times with 0.9 percent NaCl. Red cells containing methemoglobin were prepared by suspending packed red cells in an equal volume of 0.5 percent $NaNO_2$, 0.45 percent NaCl and incubating for 10 min at 25 °C. The red cells were washed five times with 0.9 percent NaCl to remove the nitrite. Red cells containing carbonmonoxyhemoglobin were prepared by blowing CO over a 25 percent (v/v) red cell suspension in Krebs-Ringer phosphate buffer (120 mM NaCl/4.7 mM KCl/1.3 mM $CaCl_2$/1.2 mM $KH_2PO_4$/1.2 mM $MgSO_4$/16.2

mM $NaH_2PO_4$, pH 7.4) until the visible spectra of red cell lysates reached a maximum at 569 nm. Red cells containing carbonmonoxyhemoglobin were then collected by centrifugation and resuspended in aerated Krebs-Ringer phosphate buffer. All incubations of red cells containing carbonmonoxyhemoglobin were carried out in stoppered flasks under an atmosphere of air/CO (9 : 1, v/v).

*Incubation conditions.* Incubations were carried out using 10 ml Erlenmeyer flasks containing 1 ml of 25 percent (v/v) red cell suspension in Krebs-Ringer phosphate buffer. Flasks were sealed with rubber stoppers and incubated in a shaking water bath at 37 °C for 1 h. *t*-Butyl hydroperoxide (2 to 8 mM) (Sigma, St. Louis, MO) was always added last after all other additions to the incubation mixture. Other variable additions were 5 mM glucose, 40 mM D-glyceraldehyde (Sigma), 40 mM L-glyceraldehyde (Sigma), 0.1 mM or 10 mM L-ascorbate (Sigma), 0.05 mM diethylenetriamine pentaacetic acid (DETAPAC) (Sigma), 0.5 mM or 5 mM deferoxamine mesylate (CIBA, Basel, Switzerland), 0.25 mM 2,6-di-*tert*-butyl-4-methylphenol (butylated hydroxytoluene) (Aldrich, Milwaukee, WI), 0.25 mM diphenylamine (Eastman Kodak, Rochester, NY) and 173 mM isopropanol (Fisher, Fair Lawn, NJ). Butylated hydroxytoluene and diphenylamine were added to the incubation mixture as stock solutions dissolved in isopropanol. An equivalent amount of isopropanol (final concentration of 173 mM) had no effect on electrophoretic patterns of red cell membrane proteins.

*Preparation of red cell ghosts and SDS-polyacrylamide gel electrophoresis.* After incubations, red cell suspensions were diluted with 0.9 percent NaCl, centrifuged (5 min at 3000 × *g*), and red cell pellets were washed once with 0.9 percent NaCl. One volume of packed red cells was lysed in 20 vol. of 5 mM $NaH_2PO_4$ (pH 8) and ghosts were washed three times with 5 mM $NaH_2PO_4$ (pH 8). Red cell ghosts were dissolved to a concentration of about 1 mg protein/ml in 1 percent sodium dodecyl sulfate (SDS), 140 mM $\beta$-mercaptoethanol, 10 percent (v/v) glycerol, 63 mM Tris-HCl (pH 6.5), and incubated at 95 °C for 5 min followed by removal of undissolved material by centrifugation (10 min at 6000 × *g*). SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli [10]. Measurement of protein concentration and staining of protein bands with Coomassie blue or by the periodic acid-Schiff reagent (PAS) procedure was carried out as described by Fairbanks et al. [11]. The numbering system for the periodic acid-Schiff bands (1 through 4) was as described by Furthmayr and Marchesi [12]. Molecular weight standards for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Richmond, CA).

*Preparation and incubation of red cell cytoplasm.* Red cells were washed three times with 0.9 percent NaCl, followed by lysis in 10 vol. of 5 mM $NaH_2PO_4$ (pH 7.4). Lysates were centrifuged (10 min at 6000 × *g*) and the red cell ghosts were discarded. Incubations were carried out in 10 ml Erlenmeyer flasks containing 1.25 ml of supernatant and 0.25 ml of other variable additions dissolved in 5 mM $NaH_2PO_4$ (pH 7.4). Flasks were sealed with rubber stoppers and incubated in a shaking water bath at 37 °C. After incubation flask contents were diluted in buffer containing SDS and $\beta$-mercaptoethanol and prepared for SDS-polyacrylamide gel electrophoresis as described for red cell ghosts.

## Results

*Effects of t-butyl hydroperoxide on oxy-, met-, and carbonmonoxyhemoglobin-containing red cells*

Fig. 1 shows SDS-polyacrylamide gel electrophoresis of membrane proteins from red cells containing oxy-, met- and carbonmonoxyhemoglobin exposed to 4 mM *t*-butyl hydroperoxide at 37 °C for one hour. In all three cell types, there was a generalised decrease of all major cytoskeletal proteins bands stained with Coomassie blue, a definite loss of band 4.2, formation of high molecular weight material (> 240K), increased hemoglobin binding, the appearance of broad bands below the 66K, 45K and 31K standards, and a generalised increase in background staining. The decrease of all major cytoskeletal protein bands was most apparent in methemoglobin-containing red cells, especially spectrin bands 1 and 2. Methemoglobin-containing red cells also showed intensification of the broad bands below the 66K, 45K and 31K standards and increased hemoglobin binding relative to oxy- and carbonmonoxyhe-

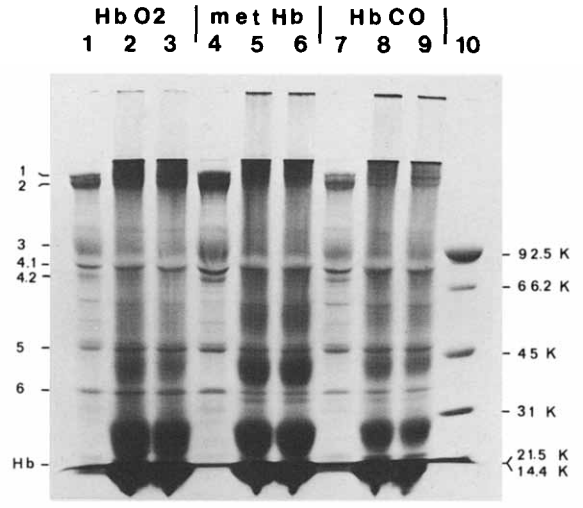


Fig. 1. SDS-polyacrylamide gel electrophoresis of membrane proteins from red cells containing oxy-, met- and carbonmonoxyhemoglobin exposed to 4 mM *t*-butyl hydroperoxide at 37 °C for 1 h. 100 μg protein was layered on each column. Columns 1, 4 and 7, controls. Columns 2, 5 and 8, red cells incubated with 4 mM *t*-butyl hydroperoxide. Columns 3, 6 and 9, red cells incubated with 5 mM glucose plus 4 mM *t*-butyl hydroperoxide. Column 10, molecular weight standards: phosphorylase b, 92500; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500; lysozyme, 14400.

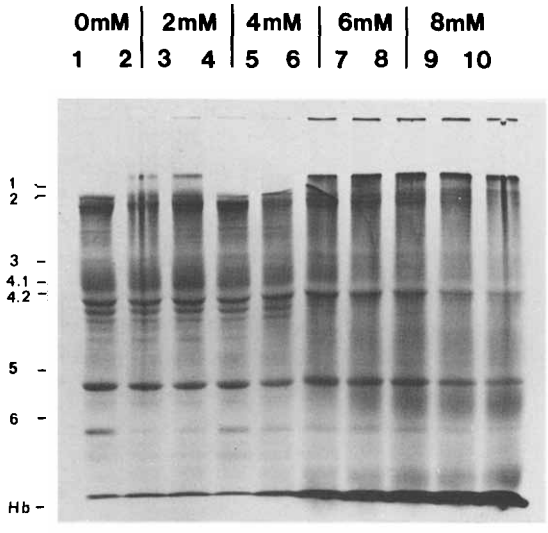


Fig. 2. SDS-polyacrylamide gel electrophoresis of membrane proteins from red cells exposed to varying concentrations of *t*-butyl hydroperoxide (0 to 8 mM) at 37 °C for 1 h. Columns 1, 3, 5, 7 and 9, red cells incubated in the absence of glucose. Columns 2, 4, 6, 8 and 10, red cells incubated in the presence of 5 mM glucose.

moglobin-containing red cells. Glucose partially prevented membrane protein changes induced by *t*-butyl hydroperoxide in oxy- and carbonmonoxyhemoglobin-containing red cells but not in methemoglobin-containing red cells.

*Concentration dependence of t-butyl hydroperoxide-induced changes in red cell membrane proteins*

Exposure of red cells to 2 mM *t*-butyl hydroperoxide (Fig. 2) caused only increased hemoglobin binding and the appearance of two broad bands below 45K and 31K (compare to Fig. 1). At 4 mM *t*-butyl hydroperoxide these new bands were more intense and were accompanied by the appearance of a broad band below 66K, formation of high molecular weight material (> 240K), loss of band 4.2 and a generalised decrease in all other major cytoskeletal protein bands. At 6 and 8 mM *t*-butyl hydroperoxide, these changes were further intensified. Glucose partially prevented all the observed effects (except for the loss of band 4.2) at 2, 4 and 6 mM, but not at 8 mM *t*-butyl hydroperoxide. Above 10 mM *t*-butyl hydroperoxide, background staining of the gel predominated and the red cells became increasingly resistant to lysis and to dissolution in the SDS buffer (data not shown).

In Fig. 3, the effects of glyceraldehyde on red cell membrane proteins are compared to the effects of *t*-butyl hydroperoxide. The specificity of the effects of glyceraldehyde (formation of high molecular weight material (> 240K), diminution in the intensity of spectrin bands 1 and 2, loss of band 6) contrasts with the generalized decrease of all major cytoskeletal bands caused by exposure to *t*-butyl hydroperoxide. In our experiment glyceraldehyde did not cause formation of new low molecular weight bands. Using different experimental conditions, Nigen and Manning [13] observed similar effects of glyceraldehyde but with the addition that at their highest glyceraldehyde concentration (20 mM) a new broad band was found at about 31K.

A duplicate to the gel shown in Fig. 3 stained by the periodic acid-Schiff reagent procedure revealed increased formation of high molecular weight material (> 240K) and increased hemoglobin binding but no change in the periodic acid-

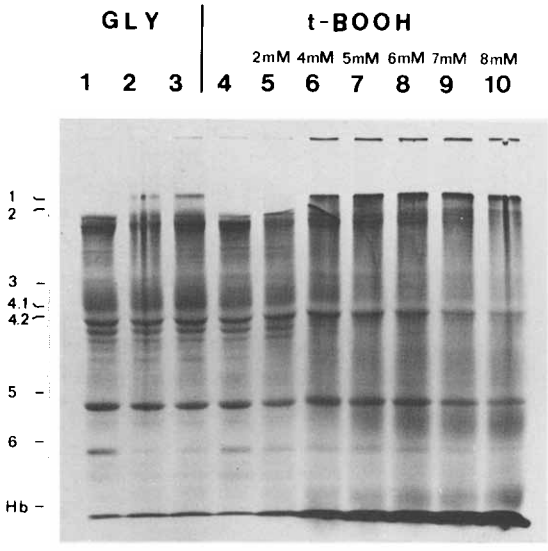


Fig. 3. Comparison of the effects of glyceraldehyde (GLY) and *t*-butyl hydroperoxide (*t*-BOOH) by SDS-polyacrylamide gel electrophoresis of red cell membrane proteins. Red cells were incubated in the presence of either glyceraldehyde or *t*-butyl hydroperoxide at 37 °C for 1 h. Columns 1 and 4, controls. Column 2, 40 mM D-glyceraldehyde. Column 3, 40 mM L-glyceraldehyde. Columns 5 through 10, 2 to 8 mM *t*-butyl hydroperoxide as indicated in the figure.

Schiff bands 1, 2, 3 and 4 with increasing concentrations of *t*-butyl hydroperoxide (data not shown). The finding of no change in the usual periodic acid-Schiff bands suggests that the generalized decrease of all major cytoskeletal bands seen with Coomassie blue stain results from formation of high molecular weight material (> 240K).

*Effects of antioxidants and chelators*

No further change in membrane protein bands was observed in red cells exposed to 4 mM *t*-butyl hydroperoxide in the presence of 0.25 mM butylated hydroxytoluene or 0.25 mM diphenylamine incubated with or without glucose, 0.1 mM or 10 mM ascorbate, 0.5 mM or 5 mM deferoxamine mesylate and 0.05 mM DETAPAC.

*Cytoplasmic hemoglobin as the source of the broad bands below 66K, 45K and 31K*

Fig. 4 shows SDS-polyacrylamide gel electrophoresis of cytoplasmic proteins after supernatants obtained by centrifugation of red cell lysates were incubated with *t*-butyl hydroperoxide. Broad bands

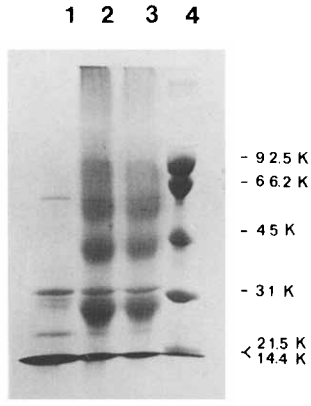


Fig. 4. SDS-polyacrylamide gel electrophoresis of cytoplasmic proteins exposed to *t*-butyl hydroperoxide. Supernatant obtained by centrifugation of red cell lysates were incubated with *t*-butyl hydroperoxide. Column 1, control. Column 2, supernatant incubated with 20 mM *t*-butyl hydroperoxide at 37 °C for 30 min. Column 3, supernatant incubated with 40 mM *t*-butyl hydroperoxide at 37 °C for 5 min. Column 4, molecular weight standards as described in the legend to Fig. 1.

are seen below 66K, 45K and 31K standards and a more diffuse and lighter band is seen at about 92K after exposure to either 20 mM *t*-butyl hydroperoxide for 30 min at 37 °C or exposure to 40 mM *t*-butyl hydroperoxide for 5 min at 37 °C. Since hemoglobin is the predominant protein in the lysate, these bands represent polymers formed by crosslinking of monomeric hemoglobin or globin chains. A similar finding was observed in hemoglobin preparations that were photo-oxidised [14].

## Discussion

Cytoskeletal protein changes have been observed in *t*-butyl hydroperoxide-treated red cells [2] and red cell ghosts [3]. In red cells, the high molecular weight material increased with increasing concentrations of *t*-butyl hydroperoxide, while it was specified that the high molecular weight aggregate was not associated with a decrease in a particular membrane protein [2]. In ghosts, similar findings to those in red cells were also reported except for the impression that band five did not change in intensity and in addition a new protein band appeared in the low molecular weight region of the gel [3]. In the current study, we observed a generalised decrease of all major cytoskeletal pro-

tein bands (but not the glycoproteins PAS 1 through 4), with the formation of high molecular weight material and increased hemoglobin binding. We also noted the appearance of three bands in the low molecular weight region of the gel (the lowest molecular weight band corresponds to the new band observed in the ghost study [3]) and the disappearance of band 4.2. The disappearance of band 4.2 can also be seen at the highest concentration of *t*-butyl hydroperoxide used in the red cell study [2], but no mention was made of this by the authors. The fact that band 4.2 did not disappear on gels of the cytoskeletal proteins of ghosts [3] is related to the analytical procedure. In the experiments with ghosts, since samples for electrophoresis were drawn directly from the incubation medium, proteins present in solution (not bound to ghosts) also appeared on the gels.

*t*-Butyl hydroperoxide is metabolised by the hexose monophosphate shunt and can be consumed by reaction with hemoglobin [4–6]. The effect of glucose in partially preventing changes in cytoskeletal membrane proteins was consistent with previous observations of glucose protection against hemoglobin degradation and binding of hemoglobin to the membrane [4–6]. In both experiments, methemoglobin-containing red cells showed no significant evidence of glucose protection, while partial protection was observed with oxy- and carbonmonoxyhemoglobin-containing red cells. Furthermore, changes in membrane proteins, hemoglobin degradation and binding of hemoglobin to membranes was greatest in methemoglobin-containing red cells. These findings are consistent with the hypothesis that the reaction with methemoglobin accounted for most of the consumption of *t*-butyl hydroperoxide in methemoglobin-containing red cells, while in oxy- and carbonmonoxyhemoglobin-containing red cells, the metabolism of *t*-butyl hydroperoxide by the hexose monophosphate shunt was important in the protection of cytoskeletal and cytoplasmic proteins.

Lipid peroxidation occurs in red cells incubated with *t*-butyl hydroperoxide. The extent of the lipid peroxidation is dependent on the liganded state of hemoglobin and on glucose metabolism [4–6]. Lipid peroxidation induced by *t*-butyl hydroperoxide is inhibited by butylated hydroxytoluene, diphenylamine or ascorbate [5,6]. The inability of these agents to prevent membrane protein changes suggests that the mechanism for inducing protein changes are different from those that produce lipid peroxidation. We have previously shown that lipid peroxidation could be almost completely inhibited by butylated hydroxytoluene with only minor effects on hemoglobin degradation and metabolism of *t*-butyl hydroperoxide by the hexose monophosphate shunt [6]. The lack of effect of DETAPAC and deferoxamine would indicate that a metal cation-mediated reaction is not responsible for the observed membrane protein changes. A similar lack of effect of either butylated hydroxytoluene or deferoxamine on cytoskeletal protein changes was observed in ghosts exposed to *t*-butyl hydroperoxide [3].

Exposure of red cells to *t*-butyl hydroperoxide resulted in crosslinking of hemoglobin subunits and probable crosslinking of cytoskeletal proteins as suggested by the diminution in intensity of the protein bands and the parallel increase in high molecular weight material. Membrane glycoproteins were not crosslinked. The *t*-butyl hydroperoxide-induced protein change was partially prevented by metabolism of *t*-butyl hydroperoxide by the hexose monophosphate shunt. Cytoplasmic and membrane protein changes were not dependent on *t*-butyl hydroperoxide-induced lipid peroxidation.

The chemical mechanism of *t*-butyl hydroperoxide-induced crosslinking is unknown. Although formation of products of lipid peroxidation can be inhibited without interfering with crosslinking this does not exclude the possibility that intermediates normally involved in lipid peroxidation (peroxides, carbonyl compounds) might be formed at sites close to reactive protein residues [3]. Amino acid residues potentially involved in crosslinking include histidine, tyrosine, tryptophan, methionine and cysteine, residues known to undergo photochemical- or ozone-induced oxidation or crosslinking [3,14]. The fact that inhibition of lipid peroxidation does not block crosslinking and that glycoproteins, which are inserted in the lipid bilayer, are not crosslinked, suggests that crosslinking reactions occur in the cytoskeleton and the cytosol but not in the lipid bilayer.

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