*Biochimica et Biophysica Acta* 854 (1986) 169–183
Elsevier



BBA 72869

# Leak formation in human erythrocytes by the radical-forming oxidant *t*-butylhydroperoxide

B. Deuticke *, K.B. Heller and C.W.M. Haest

*Abt. Physiologie, Medizinische Fakultät der Rheinisch-Westfälischen Technischen Hochschule, Pauwelsstrasse, D-5100 Aachen (F.R.G.)*

(Received July 8th, 1985)

Key words: Membrane damage; Lipid peroxidation; Membrane permeability; Membrane pore; Antioxidant; (Human erythrocyte)

**Oxidative damage of human erythrocytes by the lipoperoxide analogue, *t*-butylhydroperoxide, has been characterized with regard to ion-permeable leaks formed in the membrane. The formation of these leaks is not correlated with oxidative denaturation of hemoglobin and its precipitation at the membrane. It is also not related to the oxidation of membrane protein SH-groups. A close, although not simply proportional correlation could be demonstrated between leak formation and phospholipid peroxidation as monitored by occurrence of malondialdehyde. The two processes showed similar dependences on exposure time, concentration and temperature. Both were stimulated by the addition of azide as a ligand of ferric heme iron, and suppressed by the anti-oxidant, butylated hydroxytoluene. The leak pathway permits solute permeation with a temperature dependency of bulk diffusion in water and discriminates nonelectrolytes according to size. Discrimination among alkali chlorides corresponds to their free solution mobility; sodium halides are discriminated more effectively. Apparent radii of about 0.5–0.7 nm can be assigned to the defects, while apparent numbers of defects per cell as low as 0.1–0.2 suggest that the defects are dynamic in nature.**

## Introduction

Oxidative injury of cells by radicals is a subject of paramount interest in contemporary biological and biomedical research. The questions of which radicals are involved, which are their structural targets and which processes finally cause the death of cells and tissues subjected to this injury are being investigated in many aspects [1–6]. Membranes belong to the cell constituents most sensitive to oxidative damage, due to their content of unsaturated lipids. Formation of membrane leaks and osmotic cell lysis are therefore claimed by many investigators to be a decisive step in, if not the primary reason for, oxidative cell destruction [1–8]. The formation of these leaks, their structural organisation and their permeability properties, however, have so far not received much attention.

In the course of a comparative study of membrane leaks resulting from chemical modification of human erythrocytes [9–11], we have now studied the defects induced by the organic hydroperoxide *t*-butylhydroperoxide (*t*-BuOOH). Its action on mammalian erythrocytes was already studied by a number of investigators [12–20]. *t*-BuOOH upon entering the cell is transformed into its alkoxy-radical *t*-BuO$^{\cdot}$ by reacting with hemoglobin or methemoglobin [13–16]. The radicals attack hemoglobin producing methemoglobin and hemichro-

* To whom correspondence should be addressed.

Abbreviations: *t*-BuOOH, *t*-butylhydroperoxide; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

mes, and oxidize membrane lipids inducing formation of lipoperoxides and their degradation products [14,15,19]. Furthermore, membrane proteins aggregate in *t*-BuOOH-treated cells [12,17,18], possible causes being either direct alterations by *t*-BuO$^{\cdot}$ or modification by lipoperoxides and their degradatives.

Rate and extent of the reaction with cytoplasmic and membrane constituents are largely dependent on (a) the ligand state of hemoglobin, and (b) the metabolic situation of the cell, in particular the availability of substrate [13–15]. Protection against the oxidative attack is provided by enzymatic inactivation of *t*-BuOOH by glutathione peroxidase and GSH, and by scavengers of oxygen radicals, among which hemoglobin degradatives, thiols, ascorbate and tocopherol are of biological origin and relevance [15,18,19]. Synthetic phenolic antioxidants such as butylated hydroxytoluene have been used as tools to discriminate the various steps in the chain of peroxidative events initiated by *t*-BuOOH [15,16].

Lysis of *t*-BuOOH-treated erythrocytes, which has been described in qualitative terms [20], suggests the formation of membrane leaks. We have now characterized these leaks by measuring fluxes of probe molecules under non-lytic incubation conditions. The formation of leaks was correlated with the other effects of *t*-BuOOH treatment. The results exclude SH-oxidation and hemoglobin precipitation on the membranes as the reason of leak formation. They reveal a relationship between lipid peroxidation and leak formation but do not yet allow establishment of the mechanisms of leak formation.

## Materials and Methods

*Materials*

*t*-Butylhydroperoxide, 1,1′,3,3′-tetraethoxypropane and *N*-ethylmaleimide were purchased from Fluka, Neu-Ulm. Butylated hydroxytoluene, cytochalasin B and diamide were obtained from Sigma, Taufkirchen. $NaN_3$ and iodoacetate were purchased from Merck, Darmstadt. Dextran 1 (1 kDa) was obtained as Promit® from Schiwa, Glandorf. Chloride-36 and [$^{14}$C]erythritol were obtained from Amersham-Buchler, Braunschweig, and [$^{14}$C]mannitol, and [$^{14}$C]arabinose from New England Nuclear, Dreieich. All other chemicals were of the highest purity commercially available. 4,4′-Diisothiocyanostilbene-2,2′-disulfonate (DIDS) was a kind gift from Professor K.-F. Schnell, Regensburg.

*Methods*

*Treatment with t-butylhydroperoxide.* Human red cells from fresh blood obtained at the local blood bank and anticoagulated by heparin were washed in isotonic saline and suspended in 9 vol. of a medium of the following composition (mM): KCl (80), NaCl (40), $Na_2HPO_4/NaH_2PO_4$ (10), trisodium citrate (20) (medium A). Sodium citrate served as a colloid-osmotic protectant due to its slow permeation through the induced leaks. Cells containing only methemoglobin were prepared by incubation in isotonic $NaNO_2$ for 20 min at 20°C. Subsequently, the cells were washed and also suspended in medium A. After adjustment of pH 7.4 and temperature equilibration, another 10 vol. of medium A (pH 7.4) containing *t*-BuOOH at twice the desired final concentration were added. $NaN_3$ or radical scavengers to be tested were added to the erythrocyte suspension 5 min prior to the addition of *t*-BuOOH, dissolved in small amounts of an appropriate solvent. The suspension (final hematocrit 5%) was incubated in a water-bath for the time period chosen. At the end of the incubation, butylated hydroxytoluene, dissolved in a small amount of ethanol, was added at a final concentration of 100 $\mu$M to interrupt the peroxidative chain reaction in the membrane. The suspension was chilled to 0–4°C, the cells spun down and washed twice with medium A.

*Measurements of permeability.* Leak permeabilities for small anions and nonelectrolytes were derived from tracer flux measurements as described previously [9,10]. Relative salt permeabilities were derived from the net uptake of salts through the induced leak by following the progress of colloid-osmotic lysis in isotonic salt solution as outlined elsewhere [10,11]. Briefly, cells treated with *t*-BuOOH were washed twice in medium A, suspended in 12 vol. of an isotonic (300 mosmol/l) solution of the salt to be tested and incubated at 22–23°C under gentle shaking. After appropriate periods of time, 300 $\mu$l samples were centrifuged (12000 × *g*, 30 s). Hemoglobin in the supernatant,

serving as a measure of lysis, was determined photometrically as cyanomethemoglobin and related to the total hemoglobin content of the suspension. Rates of hemolysis, defined as the reciprocal of the time required for 50% hemolysis, were normalized to the rate for NaCl medium.

*Estimation of apparent pore radii from osmotic protection studies.* (i) *Rationale:* Red cell lysis due to formation of membrane leaks to small ions is caused by the osmotic drag of intracellular impermeable solutes (hemoglobin, organic phosphates). This colloid-osmotic lysis can be prevented by addition, to the extracellular medium, of solutes capable to counterbalance the osmotic pressure of the intracellular macromolecules. Such compounds will act protectively for an unlimited period only if they have virtually no access to the leak pathway. If they permeate slowly, they will protect for a limited period only. Leak sizes can therefore be characterized on a comparative scale by testing various solutes for their capability to fully protect cells against lysis for very long time (more than 20 h).

(ii) *Experimental procedure:* Red cells were treated with *t*-BuOOH (2 mM) and azide (5 mM) for 25–55 min at 37°C. After addition of butylated hydroxytoluene and washing, they were resuspended at 20% hematocrit in a medium containing (mM): KCl (75), NaCl (35), $Na_2HPO_4$/$NaH_2PO_4$ (12.5), pH 7.4 and in addition 30 mM of either mannitol, sucrose, raffinose or Dextran 1 (1 kDa). The final osmolarity was 280 mosmol/l. The cells were incubated on a rotating mixer at low speed (40/min) at room temperature for 21 h. Hemolysis was subsequently quantified as described above.

*Quantification of lipid peroxidation and degradation.* Malondialdehyde was determined in the *t*-BuOOH-treated cell suspensions by standard procedures [21]. The absorbance $A_{532}$ was converted into amounts of malondialdehyde formed per ml cells originally suspended in the incubation medium using the initial hematocrit value and a calibration curve obtained by reacting malondialdehyde, freshly prepared from 1,1′,3,3′-tetraethoxypropane and HCl, with the reagents required.

Oxidative membrane damage was also characterized by measuring the decrease of phosphatidylethanolamine (PE) and phosphatidylserine (PS). Lipids were extracted [22], the phospholipid fractions separated by thin-layer chromatography [23] and quantified by phosphorus analysis. Levels of PE and PS were related to those of sphingomyelin, a phospholipid not undergoing oxidative degradation under our experimental conditions. Levels of phospholipid fatty acids were determined by gas chromatography after transesterification [24].

*Measurements of red cell hemoglobin and its oxidation products.* Fractional contents of $HbO_2$ and its oxidative degradation products were determined in cell suspensions before and after exposure to *t*-BuOOH, using an approach recently described by Szebeni et al. [25]. In intervals, 50 μl samples of the suspensions were diluted into 3 ml of distilled water. The absorbances at 560, 577, 630 and 700 nm were measured in a Perkin-Elmer Lambda-5 spectrophotometer. The percentage of (residual) $Fe^{2+}$ hemoglobin was calculated as described in Ref. 25.

Membrane-bound denatured hemoglobin was determined by following the increase of light scattering, at 700 nm, in ghosts prepared from erythrocytes subjected to *t*-BuOOH treatment, following a procedure introduced by Winterbourn [26]. Low amounts of membrane-bound hemoglobin were determined by measuring the absorbance $A_{415}$ of ghosts prepared by the Dodge procedure from *t*-BuOOH-treated cells and solubilized by SDS [23]. Amounts of hemoglobin obtained from these numbers were related to the non-hemoglobin protein content of the membranes.

*Modification and determination of membrane SH groups.* For blockage of SH groups, erythrocytes were pretreated (pH 8.0, 37°C, 30 min) with sodium iodoacetate or *N*-ethylmaleimide at the concentrations given in Results. The cells were then washed and immediately subjected to modification by *t*-BuOOH. Levels of membrane SH groups were determined in ghosts prepared from the cells by the Dodge procedure and solubilized by SDS as described elsewhere [23].

## Results

### *Leak formation and oxidation of cell constituents by t-BuOOH*

#### *Time and concentration dependence*

Human erythrocytes treated with *t*-BuOOH (2 mM) at 37°C become leaky to $Cl^-$ within a very short time (Fig. 1A). Chloride permeability is defined here as the $^{36}Cl^-$ permeability of cells treated exhaustively [10] with the potent inhibitor, DIDS, which abolishes all components of $Cl^-$ movement mediated by band 3 protein. The residual permeability to chloride amounts to $2 \cdot 10^{-10}$ cm · s$^{-1}$ at 0°C in control cells and is independent of $Cl^-$ concentration at least up to 150 mM [10]. *t*-BuOOH treatment under the conditions chosen enhances permeability to about $2 \cdot 10^{-8}$ cm · s$^{-1}$ within 15–20 min. A saturating value of permeability is reached at this level. The half-time of the process is about 7 min. Concomitantly, hemoglobin is completely oxidized to methemoglobin and 'non-intact' hemoglobin degradation products [13] with a half-time of about 5 min (Fig. 1B).

Malondialdehyde appears in the *t*-BuOOH-treated cells with a half-time of about 7 min at (37°C) and reaches a saturating level at 15 min (Fig. 1C). It seems reasonable to assume that leak formation as well as lipid peroxidation are terminated by the occurrence of major amounts of oxidized forms of hemoglobin, which act as radical scavengers [13]. This terminating effect on the peroxidative chain reaction can, however, be suppressed by addition of azide to the suspensions (Fig. 1A, C). In the presence of azide, leak formation starts with some delay (Fig. 1A), but subsequently increases progressively without any notable saturation up to 50 min of *t*-BuOOH treatment when considerable lysis of the cells commences. It should be emphasized that leak permeabilities were measured after the process of leak formation had been interrupted by addition of an antioxidant, butylated hydroxytoluene. Extended incubation with *t*-BuOOH after addition of the scavenger did

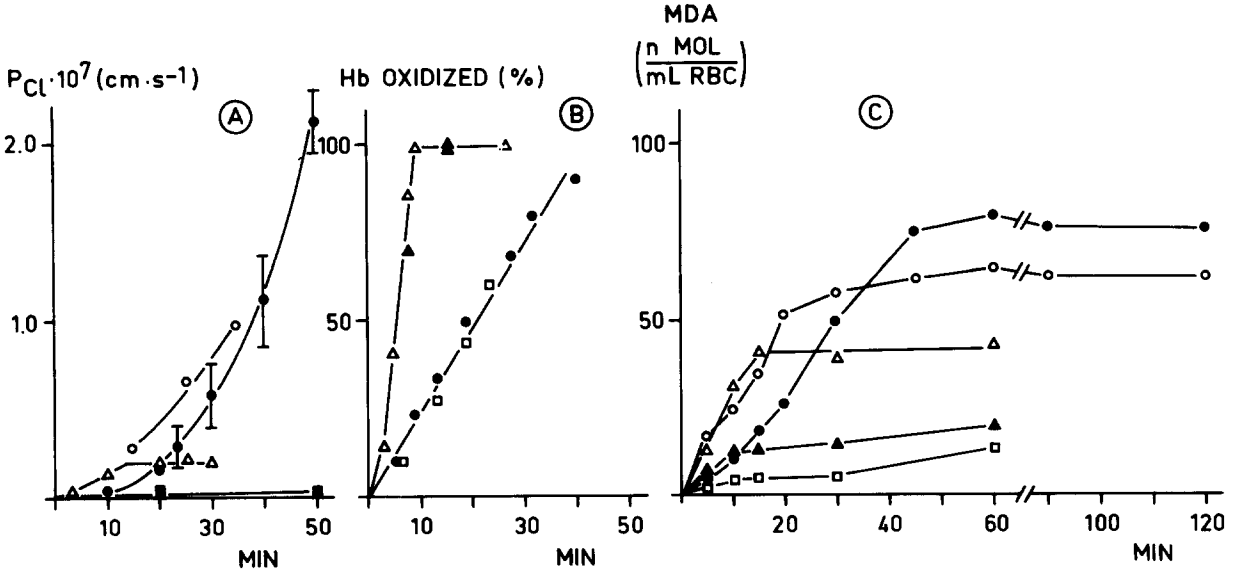


Fig. 1. Time-dependent development of *t*-BuOOH-induced damage in erythrocytes under various conditions. (A) Increase of the DIDS-insensitive $Cl^-$ leak permeability. In all cases, cells were treated (hematocrit 5%, 37°C) with 2 mM *t*-BuOOH: (△) no further additive; (●) *t*-BuOOH in the presence of 2.5 mM sodium azide; (○) *t*-BuOOH after pretreatment of the cells with isotonic $NaNO_2$ (15 min, 37°C); (□, ▲), *t*-BuOOH in the presence of 0.1 mM butylated hydroxytoluene with and without 2.5 mM azide. Exposure to *t*-BuOOH was terminated by addition of 0.1 mM butylated hydroxytoluene. The cells were washed, loaded with $^{36}Cl^-$ as described previously [9], and treated with DIDS at 0.16 μmol/ml cells [10]. (B) Oxidation of hemoglobin under the experimental conditions described under (A). For analytical details see Materials and Methods. Symbols as in (A). (C) Formation of malondialdehyde, under the experimental conditions described in (A). MDA, malondialdehyde; RBC, red blood cells.

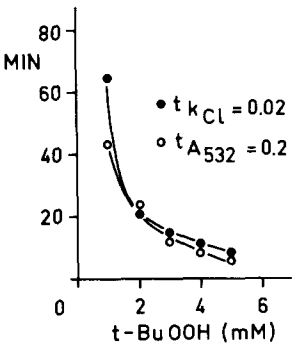


Fig. 2. Concentration dependence of the *t*-BuOOH-induced damage. Treatment of erythrocytes with varying concentrations of *t*-BuOOH in the presence of 2.5 mM $NaN_3$ leads to leak formation (measured by the chloride leak flux ($k_{Cl}$) (●)) and phospholipid degradation to malondialdehyde (determined as $A_{532}$ (○)). The time required for the rate coefficient, $k_{Cl}$, to reach a value of 0.02 $min^{-1}$ is defined as $t_{k_{Cl}=0.02}$. The time necessary to reach $A_{532} = 0.2$ is defined as $t_{A_{532}=0.2}$.

not enhance of the leak permeability any further. Azide retards the oxidation of hemoglobin (Fig. 1B). Lipid peroxidation is delayed in the presence of azide, but eventually reaches a level of saturation after 60–70 min, which is higher than that in the absence of azide (Fig. 1C).

The rate of development of the membrane damage is strongly dependent on the concentration of *t*-BuOOH. The delay time decreases progressively with increasing levels of *t*-BuOOH (at a constant level of azide). From a plot of arbitrary 'rates' of leak formation and formation of malondialdehyde the dose-response curves given in Fig. 2 are obtained. From a linearized version of this plot it can be estimated that membrane damage starts at a concentration of about 0.5 mM *t*-BuOOH at a hematocrit of 5%. This means that an amount of 10 μmol *t*-BuOOH/ml cells is required to override the innate protective mechanisms of the cell.

Variation of azide concentration reveals a biphasic effect on leak formation by *t*-BuOOH when measurements are carried out after 40 min exposure to 2 mM *t*-BuOOH (Fig. 3A). Maximal enhancement is reached between 1.25 and 2.5 mM azide. At higher concentrations, permeability decreases again. Even at 10 mM, however, leak formation is still slightly enhanced as compared to suspensions without azide.

Since malondialdehyde formation is stimulated by azide with the same concentration dependence at this fixed time of exposure (Fig. 3B), the complete time-course of leak formation at different azide concentrations is probably similar – or identical – to that of malondialdehyde formation shown in Fig. 3C. Azide delays the onset of membrane effects of *t*-BuOOH, and enhances the effects finally attained.

Leak formation is further accelerated by the

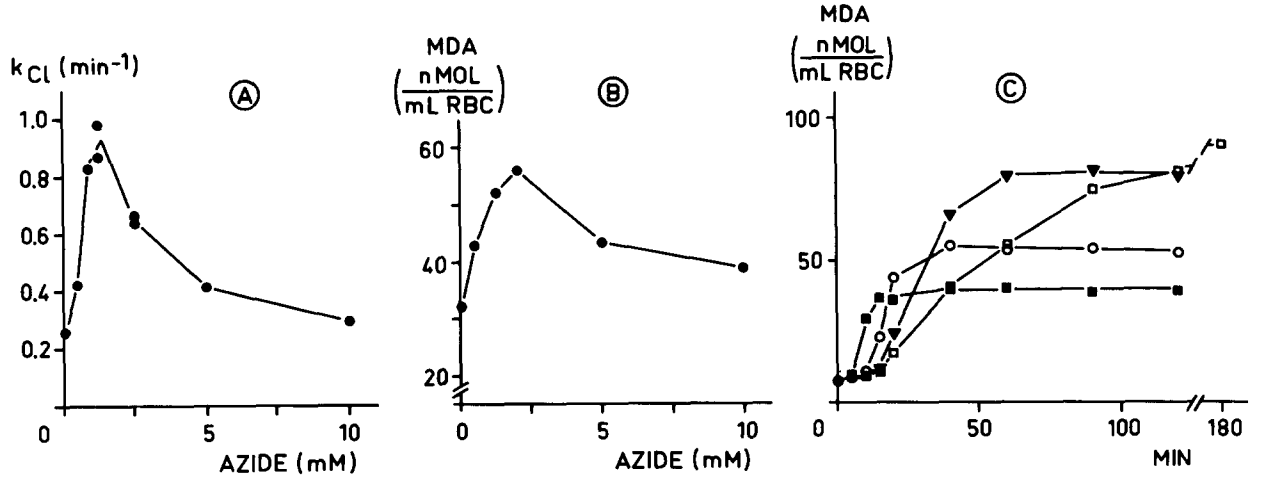


Fig. 3. Influence of azide on *t*-BuOOH-induced damage. (A) DIDS-insensitive leak flux of $Cl^-$. Cells were treated with 2 mM *t*-BuOOH for 15 min at varying concentrations of azide (37°C, pH 7.4, hematocrit 5%). For further details see Fig. 1 and Materials and Methods. (B) Formation of malondialdehyde (MDA) under the conditions described in (A). (C) Time dependence of malondialdehyde formation induced by 2 mM *t*-BuOOH at different concentrations of azide. No azide (■), 0.5 mM (○), 2.5 mM (▼), 10 mM (□). RBC, red blood cells.

presence of methemoglobin. In cells pretreated with nitrite, the delay time is considerably reduced (Fig. 1A). Formation of malondialdehyde also starts earlier but saturates at a lower level (Fig. 1C). Butylated hydroxytoluene suppresses formation of malondialdehyde (Fig. 1C), and prevents the formation of leaks (Fig. 1A) while oxidation of hemoglobin proceeds unaltered (Fig. 1B), as already observed by Trotta et al. [15].

*Influence of temperature on leak formation by t-BuOOH*

To characterize further the correlation between leak formation, oxidation of hemoglobin and lipid peroxidation, the three processes were studied at different temperatures. At $T = 15°C$, exposure to *t*-BuOOH (in the absence of $N_3^-$) induced a measurable leak permeability only after a delay of about 60 min (Fig. 4A). Leak formation increased progressively up to 120 min without reaching saturation. At 25°C, the delay time was only about 20–30 min and leak formation reached a saturation at a level about 3-times that obtained at 37°C. At low temperatures, azide, nitrite pretreatment and butylated hydroxytoluene suppressed leak formation in a concentration-dependent fashion in the time interval tested (data not shown).

The rate of oxidation of hemoglobin was also diminished at lower temperatures (Fig. 4B). Lipid peroxidation follows a pattern similar to leak formation (Fig. 4C). The reciprocal half-times of Hb oxidation and of malondialdehyde formation may be regarded as apparent rate coefficients. When these values were plotted against the reciprocal of the exposure temperature, approximately linear relationships were obtained. From the slopes an apparent (formal) activation energy of 24 kcal/mol for the *t*-BuOOH-mediated oxidation of hemoglobin and of 6 kcal/mol for the corresponding formation of malondialdehyde could be derived.

*Precipitation of oxidized hemoglobin*

Further oxidation of methemoglobin leads to the appearance of hemichromes which precipitate, giving rise, for example, to Heinz bodies. A comparison of the data compiled in Fig. 5A with those in Fig. 1 shows that precipitation of hemichromes is not releated in its time-course to malondialdehyde or leak formation. It proceeds even when these processes have been terminated spontaneously by endogenous hemichromes or are suppressed by the addition of butylated hydroxytoluene. The precipitation process is much more temperature-dependent than oxidation of hemoglobin or, in particular, malondialdehyde formation. From the slopes in Fig. 5A, a $Q_{10}$ of about 9, equivalent to an activation energy of about 50 kcal/mol, can be derived. Azide suppresses the precipitation of hemichrome in a dose-dependent way (Fig. 5B). 50% inhibition require about 0.4–0.5 mM azide (Fig. 5C). At a hematocrit of 5% this corresponds to 0.5 mol azide per mol of hemoglobin monomer. This number is reconcilable with a stoichiometric binding of azide to ferric hemoglobin. The very high affinity ($\log K = 5.4$) between azide and ferric hemoglobin [27] is also in agreement with such a quasi-stoichiometric reaction.

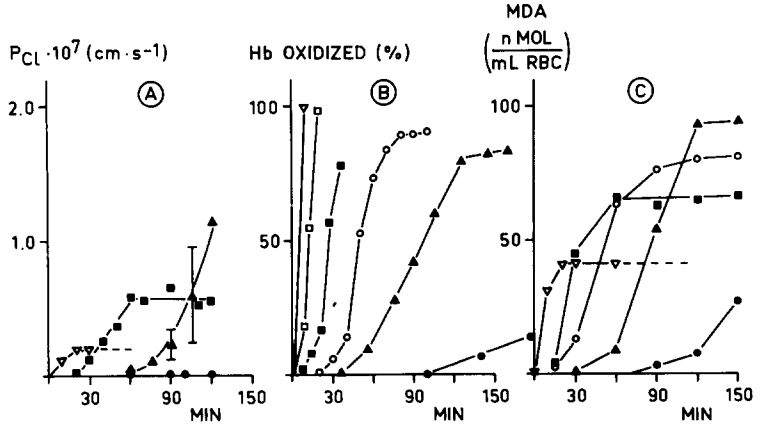


Fig. 4. Influence of the incubation temperature on the effect of *t*-BuOOH (2 mM). (A) DIDS-insensitive leak permeability for $Cl^-$. (B) Oxidation of hemoglobin. (C) Formation of malondialdehyde (MDA). ●, 10°C; ▲, 15°C; ○, 20°C; ■, 25°C; □, 30°C; ▽, 37°C. For details see Fig. 1 and Materials and Methods. RBC, red blood cells.

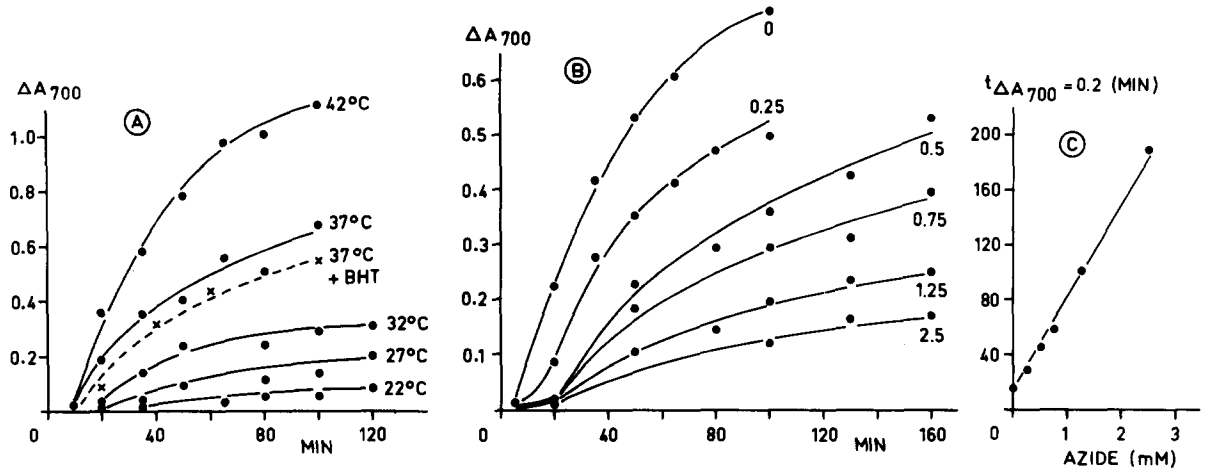


Fig. 5. Time-dependent increase of *t*-BuOOH-induced precipitation of non-intact hemoglobin. Cells suspended in medium A (hematocrit 5%) were exposed to 2 mM *t*-BuOOH. Samples were removed and diluted into 7.5 vol. of 5 mM phosphate buffer (pH 7.4)/5 mM azide to stop the progress of hemoglobin denaturation. The absorbance at 700 nm was measured before and after centrifugation for 5 min at 10000 × *g*. (A) Effect of temperature and of butylated hydroxytoluene (BHT) 0.1 mM. (B) Influence of the azide concentration (mM as indicated at the curves). Incubation at 37°C. (C) Evaluation of the data from (B). $t_{\Delta A_{700}=0.2}$ is the time required to reach an absorbance of 0.2 in (B).

*Degradation of membrane phospholipids: direct estimates*

Formation of malondialdehyde is only an indirect measure of membrane lipid oxidation which cannot be translated into fractional lipid destruction. We therefore characterized oxidative damage by following the disappearance of phospholipid fractions. Phosphatidylethanolamine rapidly disappeared from membranes treated with 2 mM *t*-BuOOH at 37°C in the presence of azide (Table I). Phosphatidylserine (and phosphatidylcholine) were much less affected. Furthermore, the level of arachidonic acid was diminished to about half its original value during 90 min of oxidative treatment at 15°C (data not shown).

TABLE I

DISAPPEARANCE OF AMINO-PHOSPHOLIPIDS DURING TREATMENT OF ERYTHROCYTES WITH *t*-BuOOH (2 mM)/AZIDE (2.5 mM) AT 37°C

PE, phosphatidylethanolamine; PS, phosphatidylserine; S, sphingomyelin, RBC, red blood cells.

| Time (min) | PE/S | PS/S | Malondialdehyde (nmol/ml RBC) |
|---|---|---|---|
| 0 | 1.0 | 0.5 | 0 |
| 25 | 0.72 | 0.42 | 47 |
| 35 | 0.66 | 0.39 | 61 |
| 50 | 0.57 | 0.38 | 83 |

*The role of SH-groups for leak formation by t-BuOOH*

In our previous studies on leaks induced by chemical modification of membranes, the oxidation of SH-groups was shown to play a major role [9–11]. In *t*-BuOOH-treated erythrocytes, the measurement of membrane SH-groups was complicated by hemoglobin attachment to the membrane occurring even in the presence of azide. A correction was made on the basis of membrane-bound hemoglobin derived from the absorbance of the solubilized membrane at 415 nm (see Materials and Methods). In these corrections, the SH-content of cytoplasmic hemoglobin, as determined in parallel, was generally used, although it is questionable that the thiols of the membrane-attached denatured hemoglobin have the same redox state as the cytoplasmic fraction [28].

Treatment of erythrocytes with *t*-BuOOH diminished the SH-content of the membrane (Fig. 6A). At 37°C in the presence of 2.5 mM azide, the measured level decreased from 90 nmol/mg protein to about 30 nmol/mg protein within 30 min.

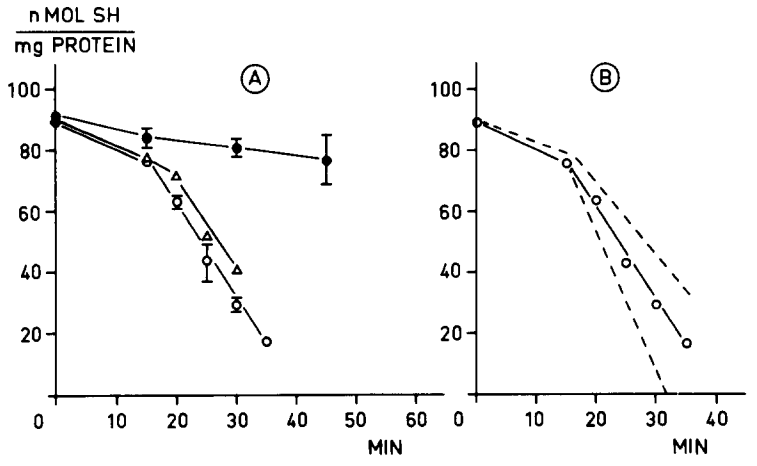


Fig. 6. Oxidation of membrane SH groups in erythrocytes treated with *t*-BuOOH. (A) Cells treated with 2 mM *t*-BuOOH at 15°C (●) or at 37°C in the presence of 2.5 mM $NaN_3$ without (○) or with (△) 0.1 mM butylated hydroxytoluene. (B) The experimental data (○) of (A), corrected for membrane-attached hemoglobin assuming either no SH-oxidation of hemoglobin (lower dashed line) or complete oxidation of SH-groups in hemoglobin (upper dashed line). For interpretation see text.

Depending on the assumptions for corrections, the true level of membrane SH-groups after the *t*-BuOOH treatment is probably somewhere between 5 and 45 nmol/mg protein (see Fig. 6B). At 15°C, where only little attachment of hemoglobin occurs (see Fig. 4B), our data suggest a very slow decrease of membrane SH-groups during the first 45 min of *t*-BuOOH treatment.

Whether the oxidation of SH-groups is causally involved in the formation of leaks does not follow conclusively from this type of data. We therefore studied the recovery of SH-groups and the changes of leak permeability upon incubation with reducing agents. Due to the *t*-BuOOH-induced attachment of hemoglobin to the membrane, quantitative data were difficult to obtain. The data shown in Table II, however, clearly indicate that the oxidized SH-groups can at least partly be recovered by a subsequent treatment of the cells with dithioerythritol. Under these conditions, the leak fluxes remain unchanged or even are somewhat enhanced. A reversible oxidation of membrane thiols is thus unlikely to contribute to leak formation.

Further support for this conclusion comes from experiments in which cells were pretreated with SH-reactive agents prior to their treatment with *t*-BuOOH. Agents such as *N*-ethylmaleimide or

TABLE II

INFLUENCE OF DITHIOERYTHRITOL (DTE, 5 mM, 37°C, 30 min) ON THE DECREASE OF MEMBRANE SH-GROUPS AND THE LEAK PERMEABILITY ($P_{Cl}$) INDUCED BY TREATMENT WITH 2 mM *t*-BuOOH AT 15°C OR AT 37°C IN THE PRESENCE OF 2.5 mM AZIDE

n.d., not determined.

| *t*-BuOOH (2 mM) | Time (min) | Before DTE | | After DTE | |
|---|---|---|---|---|---|
| | | SH (nmol/mg protein) | $P_{Cl} \cdot 10^7$ (cm·s$^{-1}$) | SH (nmol/mg protein) | $P_{Cl} \cdot 10^7$ (cm·s$^{-1}$) |
| 37°C, azide (2.5 mM) | 0 | 86 | 0.006 | n.d. | n.d. |
| | 25 | 34 | 0.231 | 65 | 0.260 |
| | 35 | 17 | 0.547 | n.d. [a] | 0.779 |
| | 50 | n.d. [a] | 1.575 | n.d. [a] | 1.685 |
| 15°C | 0 | 92 | 0.006 | n.d. | n.d. |
| | 60 | 83 | 0.038 | 95 | 0.037 |

[a] SH determinations impossible due to excessive attachment of hemoglobin to the membranes.

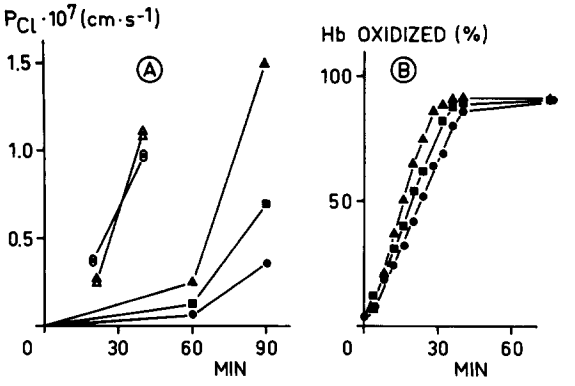


Fig. 7. Influence of SH-reactive agents on *t*-BuOOH-induced oxidative damage. (A) DIDS-insensitive $Cl^-$ leak permeability. (B) Oxidation of hemoglobin. Closed symbols, cells containing oxyhemoglobin, open symbols, cells containing only methemoglobin after pretreatment with isotonic $NaNO_2$. (●, ○) Controls, (■) cells pretreated with iodoacetate (5 mM, 30 min, pH 8, 37°C) or (▲, △) *N*-ethylmaleimide (1 mM, 30 min, pH 8, 37°C).

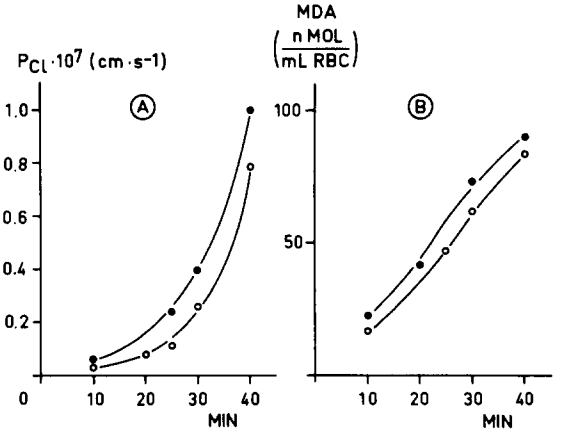


Fig. 8. Influence of glucose on *t*-BuOOH sensitivity of human erythrocytes. Cells were treated with 2 mM *t*-BuOOH in the presence of 2.5 mM azide with (●) or without 5 mM glucose (○) at 37°C. Leak permeabilities and malondialdehyde (MDA) formation measured as described in Fig. 1 and Materials and Methods. RBC, red blood cells.

iodoacetate (Fig. 7) and diamide (data not shown) did not suppress the effects of *t*-BuOOH, but even enhanced them considerably. Under the conditions of these experiments, none of the three SH-reagents in itself induces an appreciable leak permeability [9]. The stimulating effects were observed in the absence of glucose and at 15°C. This makes inhibition of metabolic reactions, normally retarding the action of *t*-BuOOH (see below), an unlikely interpretation. *N*-Ethylmaleimide pretreatment enhances the leak flux produced by *t*-BuOOH already after a brief exposure (2 min at 37°C, data not shown). Accelerated oxidation of heme iron in the hemoglobin modified by *N*-ethylmaleimide might be a reasonable explanation. This is indicated by direct measurements (Fig. 7B) and by a partial but never complete disappearence of the *N*-ethylmaleimide stimulation of leak formation by *t*-BuOOH in cells pretreated with nitrite (Fig. 7A).

On the other hand, iodoacetate, which does not modify hemoglobin to any major extent [29] also enhances the effect of *t*-BuOOH (Fig. 7A). Therefore, a modification of membrane SH-groups is probably also involved in the effects of the reagent.

*Influence of glucose metabolism on leak formation*

*t*-BuOOH is metabolized in glycolysing red cells by glutathione peroxidase [30]. This reaction produces GSSG, which can be reduced by GSH-reductase and NADPH. Reducing equivalents are furnished by glucose metabolism. Consequently, the presence of glucose should mitigate the effect of *t*-BuOOH. As is evident from Fig. 8A, there is only a weak protective effect of glucose. In the presence of 2 mM *t*-BuOOH (and 2.5 mM azide) the progress of leak formation is delayed by about 5–7 min. The same is true for the formation of malondialdehyde (Fig. 8B). The protective capacity of the GSH-peroxidase/reductase system is obviously insufficient at this concentration of *t*-BuOOH.

*Properties of the leaks induced by t-BuOOH*

*Temperature dependence of leak diffusion*

The membrane defects formed in *t*-BuOOH-treated cells are permeable not only to small anions such as $Cl^-$ but also to $K^+$ and to somewhat larger nonelectrolytes, e.g., polyols and saccharides. It is therefore likely that aqueous channels are involved. This presumption is supported by the temperature dependence of the leak permeability. Between 0°C and 30°C we obtained a linear relationship between $1/T$ and the logarithm of leak fluxes measured at different temperatures. The slopes are reconcilable with apparent activa-

tion energies of 6.4 kcal/mol for erythritol and 1.2 kcal/mol for the $Cl^-$ leak fluxes. Both values are much lower than the activation energies for the non-mediated ('basic') permeabilities for these probe molecules (erythritol 22 kcal/mol, $Cl^-$ 17 kcal/mol [9]) and indicate diffusion through a continuous aqueous phase, i.e., a hole in the membrane.

*Apparent size of the induced hole*

In an attempt to assign an apparent size to the *t*-BuOOH-induced hole we measured permeabilities of erythritol, D-arabinose and mannitol and evaluated the data in terms of the concept of restricted diffusion [31]. The data, normalized to erythritol, are plotted against the radii of the probe molecules in Fig. 9A. The predicted relations for various equivalent hole radii are indicated by the continuous lines. The experimental data are reconcilable with radii between 0.45 and 0.50 nm after treatment with *t*-BuOOH for time periods (60 and 70 min) inducing leak permeabilities with differ by a factor of about 1.7.

In a somewhat different approach, minimal hole radii were derived from the molecular radii of nonelectrolytes capable to protect 95% of *t*-BuOOH-treated cells from colloid-osmotic lysis for a period of at least 21 h. From Fig. 9B it can be seen that with increasing exposure to *t*-BuOOH larger protectants are required. After 25 min treatment, 95% protection is still provided by sucrose ($r = 0.52$ nm), while after 45 min treatment a compound of the size of stachyose (740 Da, $r = 0.67$ nm) would be required for this purpose.

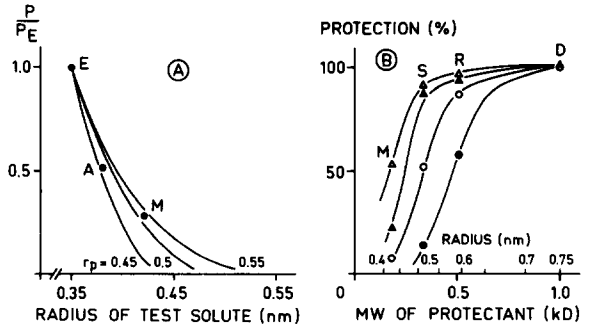


Fig. 9. Size of the leaks in erythrocytes treated with 2 mM *t*-BuOOH in the presence of 2.5 mM azide at 37°C. (A) Determination of equivalent hole radii after 60 min treatment. Leak permeabilities ($P$) for erythritol (E), D-arabinose (A) and mannitol (M) (normalized to erythritol) are plotted against the viscometric radii of the solutes. The continuous lines give the theoretical relationship between the relative permeability of compounds with increasing radii in a membrane with cylindrical pores of a given radius ($r_p$) [31]. (B) protection against colloid-osmotic lysis by nonelectrolytes of varying size. Cells were treated with *t*-BuOOH and $NaN_3$ for 25 min (△), 35 min (▲), 45 min (○) and 55 min (●). After the incubation, 0.1 mM butylated hydroxytoluene was added. The cells were washed and resuspended in phosphate-buffered saline containing mannitol (M), sucrose (S), raffinose (R) or Dextran 1 (D) (1 kDa) at a nominal concentration of 30 mM. Lysis was determined after 21 h incubation at room temperature. Apparent radii of the holes were obtained from the molecular weights ($M_r$) of protectants resulting in 95% protection against lysis. The radii were calculated according to:

$$r_x = r_{sucrose}\left(\frac{M_{r_x}}{M_{r_{sucrose}}}\right)^{1/3}$$

($r_{sucrose} = 0.52$ [9]).

*Ion selectivity*

The leaks induced by *t*-BuOOH-treatment discriminate small monovalent ions to some extent

TABLE III

RELATIVE RATES OF COLLOID-OSMOTIC HEMOLYSIS OF *t*-BuOOH-TREATED ERYTHROCYTES IN MEDIA OF DIFFERENT IONIC COMPOSITION

Cells were treated at 37°C with *t*-BuOOH (2 mM) and azide (2.5 mM) for 60 min. After washing they were resuspended in various salt media. Rates of hemolysis ($P'_{salt}$), defined as the reciprocal of the time required for 50% lysis, were normalized to NaCl. (Bulk diffusion coefficients ($D'_{salt}$) (from Ref. 11) normalized to NaCl are given for comparison.) Mean values from three experiments.

| | $P'_{salt}$ | $D'_{salt}$ |
|---|---|---|
| NaJ | 1.70 | 1.02 |
| NaBr | 1.13 | 1.02 |
| NaCl | 1.00 | 1.00 |
| NaF | 0.26 | 0.87 |
| LiCl | 0.78 | 0.84 |
| NaCl | 1.00 | 1.00 |
| KCl | 1.17 | 1.24 |
| RbCl | 1.27 | 1.27 |
| CsCl | 1.30 | 1.27 |
| Tetramethylammonium chloride | 0.52 | 0.94 |
| Tetraethylammonium chloride | 0.41 | 0.76 |
| Choline chloride | 0.40 | 0.90 |

(Table III). In case of the alkali chlorides the relative permeabilities derived from the half-times of colloid-osmotic hemolysis correspond to the relative diffusion coefficients in bulk solution. This indicates that cation-selective sites are probably not involved. In contrast, sodium halides are discriminated more than is anticipated from their relative diffusion coefficients, suggesting the presence of anion-binding sites at the entrance or within the induced leak pathway.

## Discussion

The results presented above add a further facet to the previously described effects of *t*-BuOOH, and probably other lipoperoxides, on cytoplasmic and membrane constituents of the erythrocyte. We could demonstrate formation of membrane leaks within a time regime similar to that of other alterations inflicted on red cells by the oxidants.

To achieve an unambiguous evaluation, leak formation was terminated before leak permeability was measured. A further decisive prerequisite for the interpretation was the measurement of time-courses. Changes of the experimental conditions (temperature, concentration) almost inevitably affect not only the time delay prior to the onset of the formation of malondialdehyde and of leaks but also their rates and the final levels attained. For this reason the choice of only one fixed time period for oxidative treatment may be misleading. This is particularly well illustrated by Fig. 3C, from which quite different temperature dependences of malondialdehyde formation would be derived if only one sample had been taken for analysis after, say, 20 or 90 min.

*Involvement of hemoglobin precipitation*

Precipitation of oxidized hemoglobin at the inner membrane surface is not responsible for leak formation. At 37°C, azide-liganded hemoglobin degradatives precipitate much more slowly than non-liganded forms (Fig. 4), while leak formation is markedly enhanced by azide (Fig. 1). Moreover, treatment of erythrocytes with *t*-BuOOH in the presence of butylated hydroxytoluene does not interfere with the oxidation of hemoglobin (as already reported by Trotta et al. [15]) while almost completely suppressing leak formation.

Addition of azide to cell suspensions exposed to $H_2O_2$ or organic hydroperoxides has previously been shown to stimulate the development of oxidative damage (cf., for example, Refs. 20, 21 and 32). In the case of $H_2O_2$ the stimulation has been assigned to the well-established inhibition of catalase by this anion [21]. It seems quite likely, however, that complex formation with ferric iron in hemoglobin is at least as important for the stimulation of oxidative effects.

An interesting aspect concerning hemoglobin precipitation becomes evident from comparing Figs. 1 and 5. At 37°C in the absence of azide, *t*-BuOOH-induced leak and malondialdehyde formation saturate at a low level after about 15–20 min. Membrane attachment of denatured hemoglobin, however, proceeds for at least 100–120 min. This indicates that *t*-BuO$^{\cdot}$ radicals are still formed but are captured, presumably by degrading hemoglobin [13], and therefore unable to reach or react with the sites at which lipids are oxidized and leaks formed.

*Involvement of SH-oxidation and protein modifications*

A causal involvement of *t*-BuOOH-induced oxidation of membrane SH-groups in leak formation is also unlikely. Leak formation is not reversible upon reductive treatment of the cells with dithioerythritol. This excludes disulfide bonds but does not discard irreversible oxidation of SH-groups, e.g., to sulfonate or sulfenate. A causal role of such modifications, however, can be excluded by the observations in Fig. 6. Pretreatment of the cells with SH-reagents not only does not suppress leak formation by *t*-BuOOH but even stimulates its effectivity. Under the conditions of our treatment, *N*-ethylmaleimide blocks about all of the reactive membrane SH-groups [33]. The stimulating effect of *N*-ethylmaleimide on the modification by *t*-BuOOH contrasts with its inhibitory action on leak formation by diamide or periodate, which involves formation of S-S bonds [9,11].

While SH-modification is thus unlikely to be involved in leak formation, oxidative alteration of other amino acid residues in membrane proteins has to be considered. Protein damage has been demonstrated in the case of photo-oxidative le-

sions in erythrocytes [34,35] and in a number of isolated systems [1,36]. In the case of radicals arising from the activation of *t*-BuOOH, no conclusive evidence for a direct attack on membrane proteins is available as yet. SDS gel electrophoresis indicates aggregation of cytoskeletal and other membrane proteins (band 3, band 4.2) [12,17,18]. These alterations, however, are insensitive to butylated hydroxytoluene [17,18], which completely suppresses leak formation. Protein alterations involved in leak formation therefore would have to be more subtile in nature.

*Involvement of lipid peroxides*

Peroxidation of phospholipids is regarded by many investigators (see Refs. 1, 3 and 4 for reviews) as the major event underlying oxidative membrane damage. Indeed, oxidation of artificial lipid membranes destroys the barrier properties of liposomes and small vesicles [7,37–39]. On the other hand, formation of membrane leaks in erythrocytes is possible without lipid peroxidation. Photo-oxidative damage [40] and oxidation by diamide [9] are most conspicuous examples for such an alteration.

As regards *t*-BuOOH-treated erythrocytes, our studies demonstrate a close relationship between leak formation and release of malondialdehyde. This becomes evident from (a) the time-course and extent of both processes as different temperatures (Fig. 4), (b) the very similar dependence of leak and malondialdehyde formation on the concentration of *t*-BuOOH (Fig. 2), (c) the identical effects of azide on both processes (Fig. 3), (d) the concomitant suppression of both processes by butylated hydroxytoluene (Fig. 1).

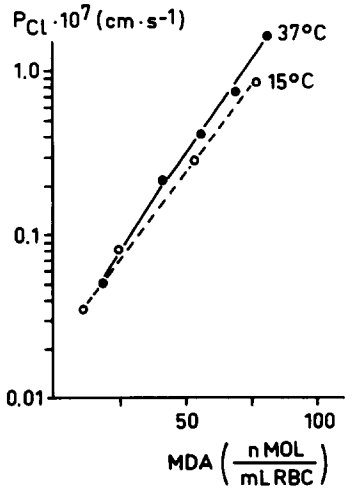


Fig. 10. Relationship between leak formation and formation of malondialdehyde (MDA) in *t*-BuOOH-treated erythrocytes. Cells exposed to 2 mM *t*-BuOOH at 15°C (○) or at 37°C in the presence of 2.5 mM azide (●). Data from Figs. 1 and 4. RBC, red blood cells.

It is difficult to decide whether these parallelisms result from a causal relationship or only reflect synchronous damage to lipids and other cellular constituents. A quantitative evaluation of malondialdehyde formation and the development of membrane leaks (Fig. 10) reveals an exponential increase of $Cl^-$ leak permeability ($P$) with the concentration of malondialdehyde ($C$) formed:

$$\log P = C \log b$$

the logarithmic form of $P = b^C$. The parameter $b$, which is a measure for the propagation of leak permeability as a function of malondialdehyde, has a value of 3. Interestingly, it is independent of the temperature and of the presence of azide. A relationship of this type suggests an avalanche type of reaction for leak formation by the structures which are the target of the oxidative damage.

Two mechanisms have to be discussed in relation to leak formation caused by peroxidation of polyunsaturated phospholipids: (1) The cleavage of alkyl chains may alter the properties of the hydrophobic membrane core at least locally. Changes of membrane fluidity [41–43] and the disappearance of aminophospholipids (Table I, and Refs. 20 and 44) support this contention. Moreover, in *t*-BuOOH-treated erythrocytes the transbilayer mobility (flip-flop) of exogenous lysophospholipids is markedly accelerated [45]. The flip sites may well be structural defects in the lipid domain also acting as aqueous leaks. (2) Two split products of polyunsaturated alkyl chains, malondialdehyde (see Ref. 46 for references) and 4-hydroxyalk-2-enals [4,47,48] have been considered as mediators of oxidative damage. Malondialdehyde may in fact contribute to leak formation. We could demonstrate somewhat enhanced leak fluxes of $Cl^-$ in erythrocytes treated with high concentrations of this compound for prolonged

periods of time (unpublished data). 4-Hydroxyalk-2-enals ($C_5$–$C_9$) react with SH-groups [48] and have been shown to lyse erythrocytes [47]. It remains to be seen whether they produce leaks of the type occurring in the *t*-BuOOH-treated erythrocytes.

*Size, number and stability of the leaks*

The leaks produced by treatment with *t*-BuOOH are comparable in their properties to the holes induced in the erythrocyte membrane by other chemical modifications [9–11]. They discriminate nonelectrolytes according to size. Two approaches suggest apparent radii of about 0.5–0.65 nm. The hole size seems to increase slightly with the length of the exposure to *t*-BuOOH (Fig. 9B). From the apparent hole radii and the induced permeabilities, apparent numbers of holes per cell can be calculated on the basis of assumptions concerning the length ($l_H$) and the diffusion coefficient ($D_H$) inside the hole. In previous studies [9,10] we have chosen 5 nm, i.e., the thickness of the lipid bilayer for the length, and used bulk diffusion coefficients. The total area available for leak diffusion ($A_{leak}$) may be calculated from these data by Eqn. 1:

$$A_{leak} = P_{Ery} \cdot A_{Ery} \cdot \frac{l_H}{D_H} \quad (1)$$

where $P_{Ery}$ = measured membrane permeability and $A_{Ery}$ = surface area of the cell ($1.4 \cdot 10^{-6}$ cm$^2$). Inserting into this equation the leak permeability for erythritol after 60 min treatment with *t*-BuOOH + azide ($P_{Ery} = 1.4 \cdot 10^{-8}$ cm $\cdot$ s$^{-1}$) one obtains a value for $A_{leak}$ of $0.2 \cdot 10^{-14}$ cm$^2$. If we accept that $A_{leak} = n \times A_{leak}$, where $A_{leak} = \pi r_H^2$, we can calculate $n$, an apparent number of holes per cell. Depending on whether we use $r_H = 0.55$ nm from Fig. 9A or $r_H = 0.67$ nm from Fig. 9B we obtain 0.1 or 0.2 holes per cell after 60 min treatment with *t*-BuOOH.

Since all cells become leaky upon treatment with *t*-BuOOH, as indicated by the extent of lysis, the number $n < 1$ suggests that either the assumptions underlying the calculations are wrong or the leaks are not static, but fluctuate with time. The assumption that bulk diffusion coefficients prevail inside the hole is certainly open to scepsis. On the other hand, it has recently been shown that even in pores smaller than our defects, the diffusion coefficients for water and small ions are not very different from their bulk values [49]. $l_H$ may be somewhat smaller than we have assumed but this will at best introduce a factor of 2–3. Consequently, we are left with the concept of a short life-time of the individual defect in cells damaged by *t*-BuOOH. This concept has already been put forward for red blood cells treated with diamide [9,10], with ionic oxidants [11] and also for cells subjected to electric breakdown of the membrane [50]. It may well account for other types of chemical or physical membrane damage, too. Further evidence for a common structure of the leaks induced by *t*-BuOOH and by other procedures may come from the ion selectivity of the defects. The sequences given in Table III qualitatively coincide with those observed for the other types of damage [9–11,50].

## Concluding remarks

The present study has provided new information on the impairment of the membrane barrier function by oxidative damage. While the oxidant used was not a physiological one there is probably little doubt that physiologically relevant radicals and radical precursors such as $O_2^-$, $H_2O_2$ will induce unspecific membrane damage [51–53] by similar mechanisms. Whether this similarity also holds for photodynamic damage remains to be seen since the role of lipid peroxidation is not quite clear (cf. Refs. 42 and 54). Although the erythrocyte is normally well-protected against such attack, certain pathological states, e.g., vitamin E deficiency [44], deficiency in the glutathione-dependent protection system [55], and deficiency in the production of reducing equivalents (NADH, NADPH) [56] will diminish the defensive capacity of the cell to a dangerous lower limit where this type of damage may become operative. The mechanism of the final destruction of oxidatively damaged erythrocytes is not yet clear. Besides a loss of rheological competence [12] and an increased sensitivity to phagocytosis possibly secondary to antibody binding induced by precipitation of hemoglobin at the membrane [57], abnormally increased ion leak fluxes may play a causal role. In other tissues the impairment of the barrier

function may be of even higher relevance [6,8]. The elucidation of the processes leading to this impairment is thus a matter of general pathobiological interest.

## Acknowledgements

The technical collaboration of Barbara Jahn, Petra Lütkemeier and Dietrun Kamp is gratefully acknowledged. We are indebted to Helga Thomas for secretarial help and to Franz-Josef Kaiser for graphic and photographic work. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 160/C3).

## References

1 Tappel, A.L. (1975) in Pathology of Cell Membranes (Trump, B.F. and Arstila, A.U., eds.), Vol. 1, pp. 145–168, Academic Press, New York
2 Petkau, A. (1982) Can. J. Physiol. Pharmacol. 60, 1327–1329
3 Halliwell, B. and Gutteridge, J.M.C. (1984) Biochem. J. 219, 1–14
4 Slater, T.F. (1984) Biochem. J. 222, 1–15
5 Younes, M. and Siegers, C.-P. (1984) Biochem. Pharmacol. 33, 2001–2003
6 Gauduel, Y. and Duvelleroy, M.A. (1984) J. Mol. Cell. Cardiol. 16, 459–470
7 Edwards, J.C., Chapman, D., Cramp, B.A. and Yatvin, M.B. (1984) Prog. Biophys. Mol. Biol. 43, 71–93
8 Dormandy, T.L. (1983) Lancet ii, 1010–1014
9 Deuticke, B., Poser, B., Lütkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210
10 Deuticke, B., Lütkemeier, P. and Sistemich, M. (1984) Biochim. Biophys. Acta 775, 150–160
11 Heller, K.B., Poser, B., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 777, 107–116
12 Corry, W.D., Meiselman, H.J. and Hochstein, P. (1980) Biochim. Biophys. Acta 597, 224–234
13 Trotta, R.J., Sullivan, S.G. and Stern, A. (1981) Biochim. Biophys. Acta 679, 230–237
14 Trotta, R.J., Sullivan, S.G. and Stern, A. (1982) Biochem. J. 204, 405–415
15 Trotta, R.J., Sullivan, S.G. and Stern, A. (1983) Biochem. J. 212, 759–772
16 Thornalley, P.J., Trotta, R.J. and Stern, A. (1983) Biochim. Biophys. Acta 759, 16–22
17 Sullivan, S.G. and Stern, A. (1984) Biochim. Biophys. Acta 774, 215–220
18 Koster, J.F. and Slee, R.G. (1983) Biochim. Biophys. Acta 752, 233–239
19 Videla, L.A., Vilena, M.I., Donoso, G., De la Fuente, J. and Lissi, E. (1984) Biochem. Int. 8, 821–830
20 Benatti, U., Morelli, A., Damiani, G. and De Flora, A. (1982) Biochem. Biophys. Res. Commun. 106, 1183–1190
21 Stocks, J. and Dormandy, T.L. (1971) Br. J. Haematol. 20, 95–111
22 Rose, H.G. and Oklander, M. (1965) J. Lipid Res. 6, 428–431
23 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
24 Deuticke, B., Grunze, M., Forst, B. and Lütkemeier, P. (1981) J. Membrane Biol. 59, 45–55
25 Szebeni, J., Winterbourn, C.C. and Carrell, R.W. (1984) Biochem. J. 220, 685–692
26 Bates, D.A. and Winterbourn, C.C. (1984) Biochim. Biophys. Acta 798, 84–87
27 Antonini, E. and Brunori, M. (1975) in Red Blood Cell (Surgenor, D. MacN., ed.), Vol. II, pp. 753–797, Academic Press, New York
28 Vilsen, B. and Nielsen, H. (1984) Biochem. Pharmacol. 33, 2739–2748
29 Benesch, R.E. and Benesch, R. (1962) Biochemistry 1, 735–738
30 Srivastava, S.K., Awasthi, Y.C. and Beutler, E. (1974) Biochem. J. 139, 289–295
31 Renkin, E.M. (1955) J. Gen. Physiol. 38, 225–243
32 Jacob, H.S., Ingbar, S.H. and Jandl, J.H. (1965) J. Clin. Invest. 44, 1187–11
33 Haest, C.W.M., Kamp, D. and Deuticke, B. (1981) Biochim. Biophys. Acta 643, 319–326
34 Dubbelman, T.M.A.R., De Goeij, A.F.P.M. and Van Steveninck, J. (1978) Biochim. Biophys. Acta 511, 141–151
35 Girotti, A.W. (1980) Biochim. Biophys. Acta 602, 45–56
36 Gardner, H.W. (1979) J. Agric. Food Chem. 27, 220–229
37 Smolen, J.E. and Shohet, S.B. (1974) J. Lipid Res. 15, 273–273–280
38 Nagatsuka, S. and Nakazawa, T. (1982) Biochim. Biophys. Acta 691, 171–177
39 Anderson, S.M. and Krinsky, N.I. (1973) Photochem. Photobiol. 18, 403–408
40 Dubbelman, T.M.A.R., De Goeij, A.F.P.M. and Van Steveninck, J. (1980) Biochim. Biophys. Acta 595, 133–139
41 Dobretsov, G.E., Borschwyskaya, T.A., Petrov, V.A. and Vladimitrov, Yu.A. (1977) FEBS Lett. 84, 125–128
42 Bruch, R.C. and Thayr, W.S. (1983) Biochim. Biophys. Acta 733, 216–222
43 Rice-Evans, C., Baysal, E., Pashby, D.P. and Hochstein, P. (1985) Biochim. Biophys. Acta 815, 426–432
44 Jacob, H.S. and Lux, S.E., IV (1968) Blood 32, 549–568
45 Haest, C.W.M., Heller, K.B., Schwister, K., Kunze, I., Dressler, V. and Deuticke, B. (1983) Biomed. Biochem. Acta 42, 127–129
46 Jain, S.K. (1984) J. Biol. Chem. 259, 3391–3394
47 Benedetti, A., Casini, A.F., Ferrali, M. and Comporti, M. (1979) Biochem. J. 180, 303–312
48 Benedetti, A., Comporti, M. and Esterbauer, H. (1980) Biochim. Biophys. Acta 620, 281–296
49 Levitt, D.G. (1984) Curr. Top. Membranes Transp. 21, 181–197
50 Schwister, K. and Deuticke, B. (1985) Biochim. Biophys. Acta 816, 332–348

51 Lynch, R.E. and Fridovich, I. (1978) J. Biol. Chem. 253, 1838–1845
52 Weiss, S.J. (1980) J. Biol. Chem. 255, 9912–9917
53 Girotti, A.W. and Thomas, J.P. (1984) J. Biol. Chem. 259, 1744–1752
54 Deziel, M.R. and Girotti, A.W. (1980) J. Biol. Chem. 255, 8192–8198
55 Kosower, N.S. and Kosower, E.M. (1978) Int. Rev. Cytol. 54, 109–160
56 Arese, P. (1982) Rev. Pure Appl. Pharmacol. Sci. 3, 123–185
57 Low, P.S., Waugh, S.M., Zinke, K. and Drenckhahn, D. (1985) Science 227, 531–533