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OXIDATIVE STRESS OF HUMAN ERYTHROCYTES BY IODATE AND PERIODATE

REVERSIBLE FORMATION OF AQUEOUS MEMBRANE PORES DUE TO SH-GROUP OXIDATION

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Human erythrocytes were exposed to oxidative stress by iodate and periodate. Oxidation causes a time- and concentration-dependent increase in membrane permeability for hydrophilic molecules and ions. The induced leak discriminates nonelectrolytes on the basis of molecular size and exhibits a very low activation energy (E_a = 1–4 kcal · mol^{-1}). These results are reconcilable with the formation of aqueous pores. The pore size was approximated to be between 0.45 and 0.6 nm. This increase in permeability is reversible upon treatment with dithioerythritol. Blocking of membrane thiol groups with *N*-ethylmaleimide protects the membranes against leak formation. The oxidation causes dithioerythritol-reversible modification of membrane proteins as indicated by the gel electrophoretic behavior. These modifications can also be suppressed by blocking the membrane thiol groups with *N*-ethylmaleimide. About half of the membrane methionine is oxidized to acid hydrolysis-stable derivatives. A fast saturating increase in diene conjugation was observed in whole cells but not in isolated membranes, with only minor degradation of fatty acid chains. The oxidation of cell membrane lipids as well as oxidation of cell surface carbohydrates are not involved in leak formation. Taken together with earlier data (Deuticke, B., Poser, B., Lütkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210), these findings indicate that formation of disulfide bonds by different oxidative mechanisms results in leaks with similar properties.

Introduction

The principal elements of biological membranes are phospholipids and proteins. Plasma membranes control solute and solvent flow to establish defined environments. This selective permeability is not an intrinsic consequence of the nature of the membrane constituents, but the result of a particular ultrastructural organization. Minor modifications may greatly perturb the mutual interaction of the constituents and thereby damage the barrier property of membranes. Most biological membranes are exposed to an oxidative environment in which various types of oxidative damage may occur, e.g., due to the formation of radicals [1,2]. Proteins may be oxidized by the reversible formation of disulfide bonds between cysteine residues or their oxidation to sulfinate, sulfenate or sulfonate [3,4], but also by irreversible modification of other amino-acid side chains, e.g., methionine, tyrosine, histidine, tryptophan [5,6]. Phospholipids containing unsaturated fatty acid chains may undergo complex transformations in-

Abbreviations: DIDS, 4,4′-diisothiocyano-stilbene-2,2′-disulfonate.

duced by oxygen radicals and leading to the cleavage of the alkyl chains after intermediate formation of conjugated double bonds [7]. One consequence of oxidative damage may be the deterioration of barrier functions. Indeed, oxidative treatments have long been known to damage artificial lipid membranes [8,9], but also to induce alterations, particularly leakiness, in biological membranes [10]. This is particularly well established for the mammalian erythrocyte [11–13]. The nature and the structural elements of the underlying leaks, however, have not been characterized.

On the other hand, leaks induced in the erythrocyte membrane by selective, reversible formation of S-S-bonds in spectrin (and other membrane proteins) by diamide have recently been studied to some detail [14,15]. It therefore seemed of interest to compare leaks induced by a more aggressive, nonspecific oxidative treatment with those occurring in the presence of diamide.

This report describes the formation and properties of aqueous membrane pores in human erythrocytes upon exposure to iodate and periodate.

Materials and Methods

Treatment of erythrocytes with iodate and periodate. Potassium iodate (Fluka) or sodium metaperiodate (Merck) were dissolved by heating in medium A (100 KCl, 50 mM NaCl, 12.5 mM Na_2HPO_4/NaH_2PO_4) at the concentrations given in Results. After cooling to the incubation temperature, the pH was adjusted to 7.4 with NaOH.

Human erythrocytes obtained at least weekly from the local blood bank and stored at 0°C were washed three times with 150 mM NaCl and the buffy coat was removed. Washed cells were suspended in medium A (10% hematocrit, 37°C) and the pH adjusted to 8.0. In some experiments, iodoacetate treatment [16] served to block the intracellular glutathione which can react with iodate and periodate.

For oxidative treatment, erythrocytes were incubated in 9 vol. iodate- or periodate-containing medium A (pH 7.4). Temperature was 37°C for iodate and 0°C for periodate-containing media, unless otherwise indicated.

At the end of the exposure, the erythrocytes were washed three times with ice-cold medium B (medium A containing 44 mM sucrose), pH 7.4 (10% hematocrit). Sucrose served to suppress colloid-osmotic hemolysis [17].

Modification of SH-groups. In some experiments, red cells were treated with *N*-ethylmaleimide (pH 8.0, 37°C, 10% hematocrit) prior to the exposure to iodate or periodate. In other experiments, the cells were treated with dithioerythritol (5 or 10 mM, pH 8.0, 37°C, 10% hematocrit) after exposure to the oxidants.

Analytical procedures. Estimation of membrane thiol groups, membrane protein contents, and SDS-polyacrylamide gel electrophoresis were performed as described elsewhere [18].

Measurement of lipid peroxidation. Malondialdehyde and other thiobarbituric acid-reactive material produced by oxidation of lipids were assayed by standard procedures [19]. For measurement of an increase in diene conjugation [20] and for gas chromatography [21], the iodate- or periodate-treated cells were washed three times with medium B (pH 7.4) and extracted [22]. The ratio A_{216}/A_{204}, determined in a Zeiss PMQ II spectrophotometer, served as a measure of diene conjugation. In untreated cells, this ratio is about 0.13 [20].

Estimation of membrane permeability. Membrane permeability was assessed by measuring the efflux of labelled test solutes from preloaded cells under self-exchange conditions as described previously [23]. Test permeants were *m*-erythritol, mannitol, arabinose, sucrose and chloride. Fluxes of the permeants which use specific transfer systems in addition to unspecific pathways were measured in the presence of appropriate inhibitors of the specific pathways. In chloride experiments, cells were exposed to 4,4′-diisothiocyano-stilbene-2,2′-disulfonate (DIDS, 0.16 $\mu mol \cdot ml^{-1}$ cells, 37°C, 30 min) after preloading the cells with $^{36}Cl^-$ and fluxes were measured in the presence of 50 $\mu mol \cdot l^{-1}$ DIDS (a kind gift of Professor F.-K. Schnell, Regensburg). The specific component of the erythritol flux [24] was suppressed by addition of 10 $\mu mol \cdot l^{-1}$ cytochalasin B (Sigma).

Net uptake of salt and water. Cells rendered leaky to otherwise impermeable electrolytes undergo colloid-osmotic hemolysis [17]. Relative rates of hemolysis determined as described elsewhere

[15] were used as a measure of leak permeability on a comparative scale.

Results

Effects of iodate and periodate on human red cell membrane permeability

Pretreatment of human erythrocytes with the oxidants iodate and periodate markedly enhances their permeability towards erythritol and chloride (Fig. 1). Since the specific transfer pathways of these two solutes [24] were blocked (by cytochalasin B and DIDS, respectively) *, the enhanced flux may be assumed to result from the formation of a leak. Periodate treatment at 0°C caused a much more pronounced increase in permeability than iodate treatment at 37°C. In fact, pretreatment with periodate at high concentrations (more than 10 mM, 60 min) or for prolonged periods (less than 10 mM, more than 120 min) induced lysis during a subsequent incubation in the absence of periodate. This lysis could be suppressed by addition of sucrose, as expected in case of colloid-osmotic hemolysis. Pretreatment with periodate at 37°C caused lysis which could only be prevented by the presence of 30 mM poly(ethylene glycol) 3000.

The periodate-induced permeability increased linearly with the exposure time up to 30 min (Fig. 2). At high concentrations of the oxidant, the rise was even overproportional. Exposing cells to increasing concentrations of periodate for 60 min also produced an overproportional increase in permeability, while after 30 min, the increase was essentially linear. Routinely, the cells were exposed to iodate and periodate at pH 7.4. For both oxidants, the increase of permeability was strongly enhanced when cells were treated at a more acid pH (Table I).

In order to establish to what extent the effect of periodate and iodate might result from a reversible oxidation of SH-groups, cells were exposed to dithioerythritol. In cells treated with periodate (5 mM, 45 min, pH 7.4), this reductive treatment rapidly led to an almost complete normalization of

* The two inhibitors are fully effective in cells treated with the oxidants.

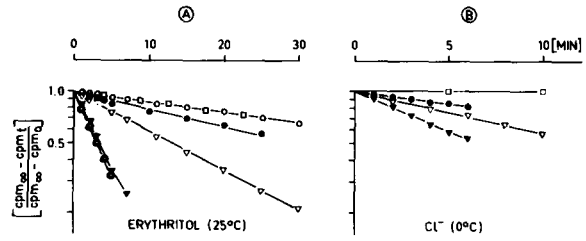

Fig. 1. Tracer efflux from control cells and cells treated with iodate and periodate. Human erythrocytes were treated with the oxidants, loaded with [^{14}C]erythritol or ^{36}Cl and resuspended in a medium containing unlabelled erythritol (at 25°C) or chloride (at 0°C). Tracer efflux was quantified as described in Materials and Methods. Rate coefficients k (min^{-1}) were derived by linear-regression analysis from the slope. (A) Controls (□); KIO_3 at 0°C (10 mM, 60 min) (○) or 37°C (●); $NaIO_4$ (5 mM, 60 min, 0°C) (▽); $NaIO_4$ (10 mM, 60 min, 0°C) (▼); $NaIO_4$ (5 mM, 10 min, 37°C) (⊛). (B) Controls (□); KIO_3 (10 mM, 60 min, 37°C) (●); $NaIO_4$ (5 mM, 60 min, 0°C) (▽); $NaIO_4$ (10 mM, 60 min, 0°C) (▼).

permeability (Table II). The leak permeability induced by 10 mM iodate was reversible to a lesser extent (60%), although the primary leak was less pronounced than that induced by periodate. These findings suggest that a reversible oxidation of thiol groups is the major reason of the increased permeability.

This conclusion is further supported by data

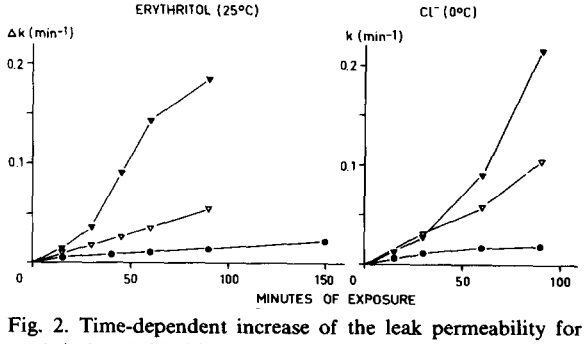

Fig. 2. Time-dependent increase of the leak permeability for erythritol and chloride upon treatment of human red cells with iodate and periodate. Cells were incubated with 10 mM KIO_3 at 37°C (●) or 5 mM (▽) or 10 mM $NaIO_4$ (▼) at 0°C. After washing, the cells were loaded with erythritol or chloride and efflux was measured as described in materials and Methods. The values for erythritol represent the increase Δk of the rate coefficient, k, over the appropriate controls. Chloride fluxes were measured at 0°C at which efflux from control cells could be neglected.

TABLE I

ERYTHR EFFLUX FROM ERYTHROCYTES TREATED WITH OXIDANT

The efflux was measured at 0°C in the presence of 10 μM cytochalasin B. Values are means ± S.D.

pH	$k \cdot 10^2$ (min^{-1})	
	Iodate (10 mM, 60 min, 37°C)	Periodate (5 mM, 45 min, 0°C)
6.0	6.46	19.40
6.5	2.27 (±0.26)	3.46
7.4	1.26 (±0.14)	2.20 (±0.27)
8.0	1.38 (±0.22)	1.80 (±0.20)

obtained with *N*-ethylmaleimide, which covalently and irreversibly binds to thiol groups. Treatment of erythrocytes with *N*-ethylmaleimide (0.4 mM, 20 s, pH 8.0) has previously been shown [14] to suppress leak formation by diamide. Such a brief pretreatment had no detectable effect on the action of periodate (data not shown). A 15 min pretreatment, however, resulted in a concentration-dependent protection against the increase of permeability for chloride and erythritol (Fig. 3), which saturated at 90%. Effects of iodate were also suppressed.

TABLE II

REVERSIBILITY OF PERIODATE- AND IODATE-INDUCED LEAK FLUXES BY SUBSEQUENT TREATMENT OF THE ERYTHROCYTES WITH DITHIOERYTHR

Dithioerythr (DTE) was 5 mM for chloride fluxes and 10 mM for erythr fluxes. Rate coefficients (min^{-1}) were normalized to those in oxidant-treated cells. n.d., not determined.

	k'	
	Chloride	Erythritol
Periodate		
5 mM, 0°C, 45 min	1.00	1.00
+ DTE (pH 8.0)		
10 min	0.18	0.07
30 min	0.15	0.03
Periodate		
5 mM, 0°C, 90 min	1.00	1.00
+ DTE (pH 8.0)		
10 min	0.14	0.11
30 min	0.11	0.11
Iodate		
10 mM, 37°C, 45 min	1.00	1.00
+ DTE (pH 8.0)		
30 min	0.45	0.33
60 min	n.d.	0.34

Properties of the leak induced by iodate and periodate

One explanation for the increased permeability of the human erythrocyte membrane after treament with iodate and periodate is the formation of aqueous leaks. Permeation through such leaks should have a low activation energy and exhibit no saturation of the fluxes with substrate concentration. Moreover, uncharged substrates should be discriminated according to their molecular size. Indeed, the rate coefficients of chloride flux did not decrease up to concentrations of 450 mM NaCl (data not shown). The apparent activation energies of the inhibitor-insensitive fluxes of erythritol and chloride in native cells were 22.4 and 16.8 kcal · mol^{-1}, in agreement with earlier measurements [14,24]. The leak permeabilities induced by iodate had apparent activation energies of 5.5 (erythritol) and 3.6 (chloride) kcal · mol^{-1}, while the apparent activation energies for the periodate-induced fluxes were as low as 3.6 and 0.6 kcal · mol^{-1}, respectively. Such data are reconcilable with diffusion through an aqueous pathway induced by the two oxidants.

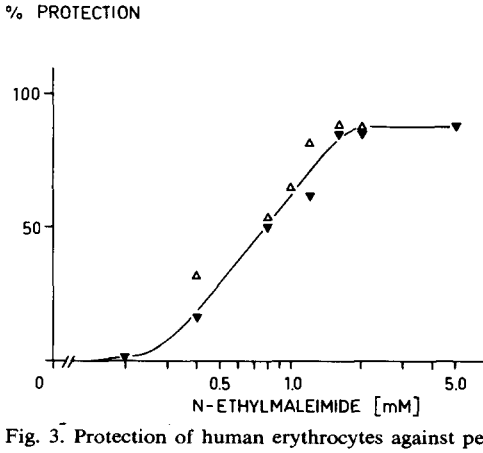

Fig. 3. Protection of human erythrocytes against periodate-induced damage by *N*-ethylmaleimide. Cells were preincubated with *N*-ethylmaleimide for 15 min at pH 8.0 at 37°C before oxidation with 5 mM periodate for 45 min at 0°C. The periodate-induced leak fluxes of [^{14}C]erythritol at 25°C (▼) and of ^{36}Cl at 0°C (△) were measured in untreated cells and cells treated with *N*-ethylmaleimide.

This assumption was supported further by measurements of the periodate-induced increase of permeability for a number of hydrophilic nonelectrolytes varying in molecular size. The leak induced by 75 min incubation with 10 mM periodate (0°C) allowed permeation of otherwise factually impermeant polyhydroxy compounds up to the size of sucrose (Table III). Permeabilities decreased with increasing radius of the probe. The data point to a minimal pore radius of 0.55–0.60 nm (see Discussion).

To characterize further the selectivity of the leak induced by the oxidants, the rates of uptake of various alkali chlorides and sodium halides were compared by measuring the rates of colloid-osmotic hemolysis of periodate-treated cells in isotonic solutions of these salts. Relative rates of hemolysis increased with increasing crystal radii (decreasing hydrated radii) of the varied ion (Fig. 4), supporting the assumption that the oxidative treatment induces a leak discriminating small electrolytes to some extent. The induced leaks discriminate between anions to a greater extent than between cations. A similar observation, made in the case of the diamide-induced leaks, proved to be due in part to an enhancement of the induced permeability by strongly absorbing (chaotropic) ions [15]. As shown in Table IV, such ions also enhanced the leak permeabilities induced by iodate and periodate.

TABLE III

NONELECTROLYTE PERMEABILITIES OF ERYTHROCYTES PRETREATED WITH PERIODATE (10 mM, 0°C, 75 min)

After the treatment, the cells were washed and loaded with labelled test solute in medium A containing 30 mM poly(ethylene glycol) 4000 as colloid-osmotic protectant. Fluxes were measured at 0°C as described in Materials and Methods. Values are means ± S.D. of at least four independent experiments.

	$P \cdot 10^8$ (cm·s^{-1})
Erythr	6.37 ± 0.78
D-Arabinose	3.15 ± 0.40
Mann	1.43 ± 0.53
Sucrose	0.21 ± 0.04

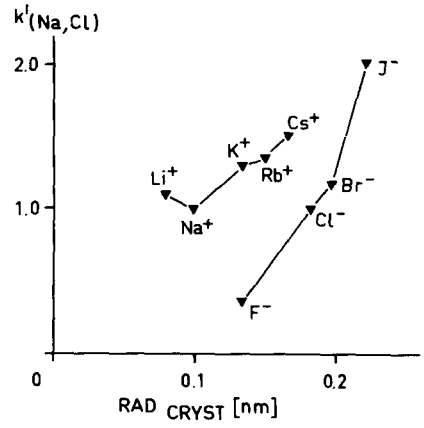

Fig. 4. Permeation of electrolytes through periodate-induced leaks. Human erythrocytes were treated with 10 mM $NaIO_4$ at 0°C for 60 min. Treated cells were resuspended in isotonic solution of alkali chlorides or sodium halides containing 3 mM phosphate buffer (pH 7.4) at 25°C. Rates of lysis were determined by measuring hemoglobin in the supernatant in appropriate intervals. The k value was defined as a reciprocal of the time needed for 50% hemolysis. The k' values (k values relative to those obtained with NaCl) are plotted against the crystalline radius of the ion species that was varied.

Modification of human erythrocyte cell membrane constituents by iodate and periodate

The leak permeability induced by treatment with diamide has been causally related to the formation of disulfide bonds and crosslinking of membrane proteins [14,15]. Since according to the

TABLE IV

INFLUENCE OF THE ION MILIEU ON CHLORIDE LEAK FLUXES FROM ERYTHROCYTES TREATED WITH OXIDANTS

Cells were exposed to the oxidants in medium A and subsequently equilibrated with isotonic solutions of the different ions (containing 12.5 mM phosphate buffer (pH 7.4) and 30 mM poly(ethylene glycol) 4000). Chloride leak fluxes were measured at 0°C as described in Materials and Methods.

	$k \cdot 10^2$ (min^{-1})	
	Iodate (20 mM, 60 min, 37°C)	Periodate (5 mM, 45 min, 0°C)
Cells equilibrated in		
NaCl	3.54	3.73
$NaNO_3$	10.88	9.43
NaSCN	5.77	6.36
Guanidine·HCl	11.43	13.04

data presented above, the more potent oxidants iodate and periodate probably cause membrane damage by a similar reaction, chemical alterations of cell and membrane constituents were studied.

Exposure of human red cells to iodate (37°C) and periodate (0°C) led to a rapid oxidation of cellular glutathione. Within 10 min, 90% of the glutathione was oxidized to glutathione-disulfide and mixed disulfides, which could be fully reduced upon treatment with dithioerythritol (data not shown). Membrane SH-groups were also readily oxidized by periodate at 0°C and by iodate at 37°C, but not at 0°C. The rates of reaction (Fig. 5) of periodate (and iodate, data not shown) with membrane SH-groups increase with decreasing pH values. About 20% of the membrane SH-groups were not available for oxidation under optimal conditions (pH 6.0). 80% of the oxidized SH-groups were recovered upon treatment with dithioerythritol. Essentially all of the reactive SH-groups are oxidized by periodate and iodate after a 60 min treatment (Fig. 5B). Interestingly, in intact cells, SH-groups were oxidized by periodate at a lower rate (Fig. 5B) than in isolated membranes (leaky ghosts), presumably due to a protective influence of hemoglobin.

Gel electrophoretic analysis of membrane proteins from periodate- or iodate-treated cells revealed conspicuous changes (Fig. 6). Exposure for 60 min induced an almost complete disappearance of most of the polypeptide bands, while high

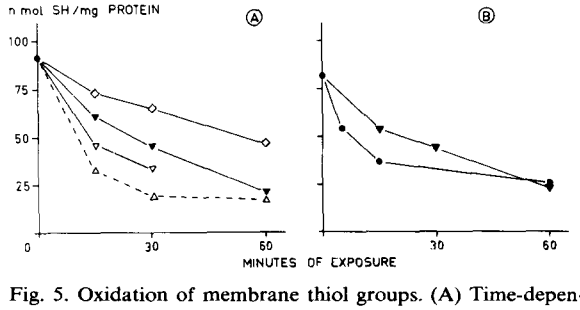

Fig. 5. Oxidation of membrane thiol groups. (A) Time-dependent oxidation of membrane SH-groups of whole cells (straight lines) and isolated membranes (dashed line) by 5 mM periodate at pH 8.0 (◇), pH 7.0 (▼) and pH 6.0 (▽,△). The experiments were carried out at 0°C. (B) Time-dependent oxidation of human erythrocyte membranes by 5 mM periodate (▼) at 0°C and 5 mM iodate (●) at 37°C. The experiments were performed at pH 7.4.

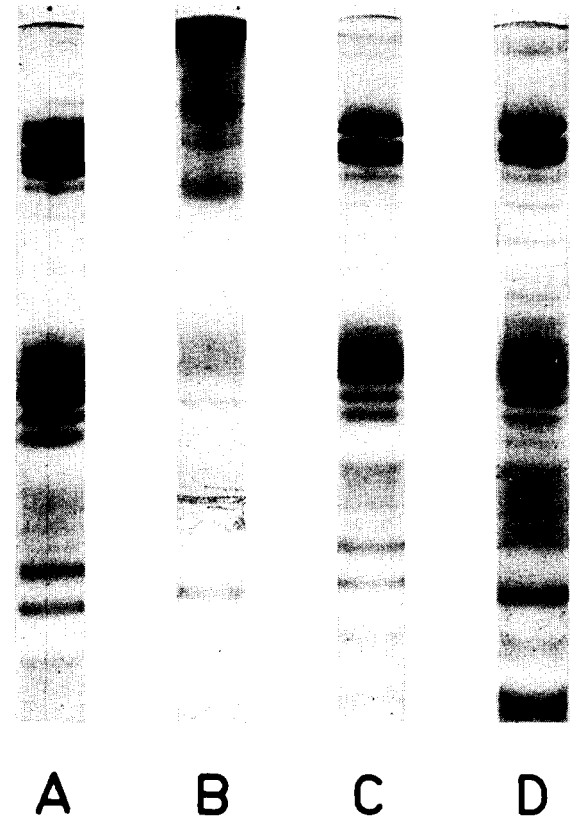

Fig. 6. Polypeptide pattern of isolated human erythrocyte membranes. Isolated membranes of control cells (lane A), cells treated with periodate (5 mM, 60 min, 0°C) (lane B), cells pretreated with *N*-ethylmaleimide (5 mM, 15 min, 37°C, pH 8.0) before exposure to periodate (5 mM, 60 min, 0°C) (lane C), and periodate-treated cells (5 mM, 60 min, 0°C) subsequently exposed to dithioerythritol (10 mM, 15 min, 37°C, pH 8.0) were submitted to SDS-polyacrylamide gel electrophoresis [17].

molecular weight material not penetrating the gel accumulated. Similar findings were previously reported by Gahmberg et al. [25] for isolated membranes treated with periodate at 22°C. Treatment with dithioerythritol led to a full restoration of the original gel pattern (Fig. 6, lane C) while pretreatment with *N*-ethylmaleimide (Fig. 6, lane D) completely prevented formation of the protein aggregates. Both observations point to a causal role of SH-groups in the formation of aggregates. This does, however, not exclude that other amino-acid residues are also oxidized by iodate or periodate. To clarify this problem, the total amino-acid com-

position of cell membranes isolated from iodate- and periodate-treated human erythrocytes was studied by routine amino-acid analysis of acid-hydrolysed membranes. The results (not shown) indicate that between 30 and 40% of the methionines were oxidized to acid-stable products with no significant difference between iodate- and periodate-treated cells. Oxidation products of cysteine were not detectable by the technique used here. Moreover, under our experimental conditions (0°C) no indication of an oxidation of tyrosine could be obtained, which was reported by Gahmberg et al. [25] for isolated spectrin treated at 22°C.

Periodate readily reacts with carbohydrates [26], which are a constituent of membrane glycoprotein. Extensive treatment of erythrocytes with trypsin or pronase cleaves off most of these carbohydrates [27] without affecting transport function. Treatment of erythrocytes with either of these enzymes before oxidation with periodate did not affect the leak formation (data not shown). Oxidation of cell carbohydrates was thus not involved in the formation of the leak.

Besides oxidation of amino-acid residues, oxidation of lipids might be relevant for the enhancement of membrane permeability. The essential stages in the oxidation of polyunsaturated lipids include migration of double bonds to give diene conjugation, followed by formation of hydroperoxides. Subsequent cleavage of the chain which produces malondialdehyde and other short-chain dialdehydes and dicarboxylic acids completes the process [7].

The level of diene conjugation in the membrane lipids increased in the presence of iodate (37°C) and periodate (0°C), reaching a saturating level after about 30 min (Fig. 7). Periodate treatment resulted in an increase about twice that obtained with iodate. Iodate treatment at 0°C did not induce diene conjugation. In isolated membranes, iodate and periodate did not cause diene conjugation, a surprising observation pointing to the involvement of cytoplasmic constituents in the oxidation of the lipids.

Formation of malondialdehyde and other thiobarbituric acid-reactive material could not be studied due to a discoloring reaction of iodate and periodate with the thiobarbituric acid reagent. Analysis of the membrane phospholipid fatty acids

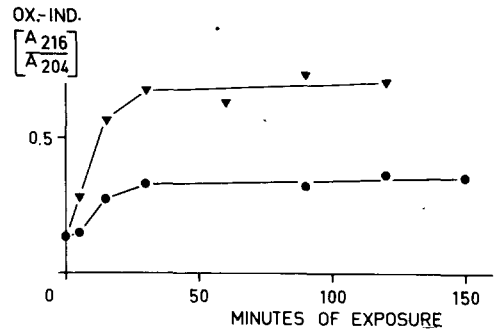

Fig. 7. Diene conjugation in human erythrocyte membranes after oxidation. Human red cells were incubated with 10 mM $NaIO_4$ (▼) at 0°C or 20 mM KIO_3 (●) at 37°C. Treated cells were thoroughly washed, lipid was extracted [21] and the absorptions were measured at the wavelengths indicated. The oxidation index (Ox.-Ind.) is a relative measure of the amount of conjugated double bonds.

by gas chromatography of the fatty acid methylesters, however, revealed only a small (6–7%) decrease of the levels of polyunsaturated fatty acids after periodate oxidation (data not shown). No indication for the oxidation of cholesterol was obtained.

Role of transmembrane uptake for the effect of iodate and periodate

To elucidate the role of transmembrane uptake for the effect of the oxidants and to characterize their pathway of uptake, leak formation and net entry – as evaluated by an indirect technique [28] – were studied in presence of an established inhibitor of anion exchange via band 3. Leak formation was blocked to at least 80% by 250 μM 4,4′-dinitro-stilbene-2,2′-disulfonate. Net entry, which could only be studied for iodate, was also found to be blocked by this inhibitor. These findings strongly suggest that the uptake of the oxidants is necessary for leak formation and that band 3 is involved in this uptake.

Discussion

The potent ionic oxidant, periodate, has long been known to modify biological membrane function at low concentrations [29,30] by cleaving C-C bonds between vicinal OH-groups in the carbohydrate moiety of glycoproteins [27]. In addition, periodate and iodate may produce membrane

damage by reacting with other membrane constituents. Effects on amino acids, particularly methionine [31] and cysteine residues, in soluble proteins have been demonstrated [4,5,25,32]. Moreover, such oxidant anions inhibit ATPase [33] and irreversibly suppress inactivation of the fast sodium channel in nerve tissue [34]. In the present report, we have shown that periodate and iodate induce the formation of leaks in the erythrocyte membrane, which may cause colloid-osmotic lysis. Loss of K^+ from periodate-treated erythrocytes has previously been described [35]. There is also evidence that periodate treatment prevents Ca^{2+} accumulation in sarcoplasmic reticulum [36], possibly due to an enhanced Ca^{2+} leak.

Properties of the leak

The induced leaks in the red cell membrane may be envisaged as aqueous pores. This is indicated by (a) the low activation energy of the leak fluxes, (b) the rather low ion selectivity of the induced pathway, (c) the nonsaturating increase of chloride leak flux with the chloride concentration, and (d) its discrimination of nonelectrolytes according to size. In these features, the leaks induced by the two oxidants are very similar to the leak induced by diamide [14]. The estimation of an equivalent cylindric pore radius for the leak by a trial and error procedure (Ref. 37, equation 11) results in a value of 0.45–0.55 nm (Fig. 8), similar to mean radii for the leak induced by diamide [14].

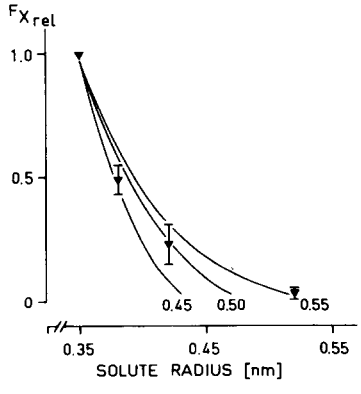

Fig. 8. Permeation of hydrophilic nonelectrolytes through periodate-induced leaks. Permeabilities taken from Table III were normalized to erythritol permeability. The continuous lines are the theoretical slopes for the expected relationship between relative permeabilities (F_x) [36] of compounds with increasing radius through cylindrical pores with the radii (nm) given at each curve.

As a further similarity, both the periodate- and the diamide-induced leaks discriminate anions and cations according to the sequence of their hydrated radii, which suggests a low affinity of the ions for their pathway of penetration [15,38]. Moreover, in both instances, number or size of the leaks are sensitive to the ion milieu (Ref. 15 and Table IV). There is thus good reason to believe that the leak produced by periodate and iodate is similar in its structural properties to that induced by diamide.

This view is also supported by the observation that in periodate-treated cells, as in diamide-treated cells, the transbilayer mobility of phospholipids, as probed by exogenous lysophospholipids, becomes enhanced considerably but reversibly [39].

Membrane modifications responsible for leak formation

The two oxidants induce leaks to a major extent as the consequence of the formation of disulfide bonds. Evidence comes from the reversibility by dithioerythritol and the suppression of the effect by *N*-ethylmaleimide. Taken for themselves, each of these two observations may not be an absolute indication, since dithioerythritol also reduces methionine sulfoxide [3,40] and *N*-ethylmaleimide may also block amino groups [40]. The combined evidence, however, is rather convincing. It excludes a major causal involvement of other alterations such as lipid oxidation or oxidation of other protein side chains, although these alterations clearly do occur.

While reversible oxidation of SH-groups is thus involved in the leak-producing effects of both periodate and diamide, the details of the modifications leading finally to leak formation are clearly different, as indicated by the following observations.

(1) Leak formation by diamide can be suppressed almost completely by blocking, with *N*-ethylmaleimide, a very small (less than 5%) fraction of the membrane SH-groups, presumably located in spectrin. The effect of periodate is only suppressed stepwise by increasing, much higher, levels of *N*-ethylmaleimide, approximately in relation to the fraction of membrane SH-groups blocked. (2) Diamide-induced SH-oxidation and leak formation are accompanied by a rather selec-

tive crosslinking of the subunits of spectrin. Only after extensive treatment, other polypeptide fractions are found in high molecular weight aggregates. In contrast, SH oxidation and leak formation observed after periodate and iodate treatment go along with reversible aggregation of all polypeptide fractions, even after short treatment with the oxidants. An aggregation of membrane proteins probably occurs already in situ in periodate-treated cells, as indicated by clustering of membrane-intercalated particles [25]. (3) Diamide produces leaks only at higher (more than 20°C) temperatures while periodate is effective at 0°C.

Both iodate and periodate enter the erythrocyte under the conditions under which they produce leaks, as indicated by the rapid oxidation of glutathione. At 0°C, iodate does neither oxidize glutathione nor membrane SH-groups in native cells, while being effective in isolated membranes at this temperature. The native membrane is thus a barrier for iodate but not for periodate at 0°C. Since both oxidants are anions of rather strong inorganic acids ($pK_{IO_3^-} = 0.95$, $pK_{IO_4^-} = 1.64$ [41]), it is not unexpected that they cross the membrane via the anion transport system in band 3. The suppression of leak formation by a potent inhibitor of anion transport indicates that the SH-groups responsible for leak formation are located inside of the anion barrier of the membrane.

In mechanistic terms, the oxidant anions would appear to act by ionic attack [26], comparable to the oxidation of soluble enzymes [31,32]. While such a simple explanation may well hold, there are preliminary observations which are difficult to reconcile with ionic oxidation. First, both oxidants affect membrane lipids in cells but not in isolated membrane. Second, we have recently observed that agents interfering with oxidation by radicals, in particular *t*-butyl-hydroxytoluene, completely suppress leak formation by periodate but not by iodate at very low concentrations. In parallel with the effect on leak formation, *t*-butyl-hydroxytoluene suppresses SH-group oxidation by periodate. The relevance of these observations is the subject of present studies.

Regardless of these mechanistic details, our results demonstrate by a further example that the barrier function of the plasma membrane is particularly dependent on the redox state of the membrane thiols, which makes the control of this state a matter of paramount relevance for cell survival.

It will be of pathophysiological interest to investigate whether membrane leaks comparable to those produced by our experimental approach also occur in erythrocytes in vivo in pathological states going along with an increased oxidative stress of the cells (glucose-6-phosphate dehydrogenase deficiency, β-thalessemia, etc. [42.43]).

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