*Biochimica et Biophysica Acta*, 731 (1983) 196–210
Elsevier

BBA 71654

# FORMATION OF AQUEOUS PORES IN THE HUMAN ERYTHROCYTE MEMBRANE AFTER OXIDATIVE CROSS-LINKING OF SPECTRIN BY DIAMIDE

B. DEUTICKE *, B. POSER, P. LÜTKEMEIER and C.W.M. HAEST

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

(Received December 8th, 1982)

*Key words: Spectrin cross-linking; Thiol group; Diamide; Membrane skeleton; Membrane permeability; Aqueous pore; (Erythrocyte membrane)*

**Oxidation of erythrocyte membrane SH-groups by diamide and tetrathionate induces cross-linking of spectrin (Haest, C.W.M., Kamp, D., Plasa, G. and Deuticke, B. (1977) Biochim. Biophys. Acta 469, 226–230). This cross-linking was now shown to go along with a concentration- and time-dependent enhancement of membrane permeability for hydrophilic nonelectrolytes and ions. The enhancement is specific for oxidative SH-group modifications, is reversible by reduction of the induced disulfides, can be suppressed by a very brief pre-treatment of the cells with low concentrations of *N*-ethylmaleimide and is strongly temperature-dependent. The pathway of the induced permeability discriminates nonelectrolytes on the basis of molecular size and exhibits a very low activation energy ($E_a$ 3–8 kcal/mol). These findings are reconcilable with the formation of a somewhat inhomogeneous population of aqueous pores with radii probably $\leqslant$ 0.65 nm. Estimated pore numbers vary with the size of the probe molecule. Assuming a diffusion coefficient as in bulk water within the pore, at least 20 pores per cell have to be postulated; more realistic lower diffusion coefficients increase that number. Alterations of the lipid domain by changes of cholesterol contents and insertion of hexanol or nonionic detergents alter the number or size of the pores. Since aggregation of skeletal and intrinsic membrane proteins also occurs after the SH-oxidation, in parallel to the formation of membrane leaks, one may consider (a) defects in the disturbed bilayer interface, (b) a mismatch between lipid and intrinsic proteins or (c) channels inbetween aggregated intrinsic proteins as structures forming the pores induced by diamide treatment.**

## Introduction

Plasma membranes are tight diffusion barriers to hydrophilic solutes in spite of their very inhomogeneous structure and composition. The permeation of ions and hydrophilic nonelectrolytes occurs with high preference via specialized and controlled pathways exhibiting the properties of carriers or channels. Unspecific leaks usually contribute little to the total permeability [1] and can often be neglected in transport studies on polar permeants – in contrast to the situation for less polar substrates, for which transport via the lipid domain of the membrane may provide considerable 'leaks' [2].

The low leak permeability to polar substrates is, however, not an intrinsic consequence of membrane composition, but the result of certain ultra-

* To whom correspondence should be addressed.

Abbreviations: DIDS, 4,4'-diisothiocyano-2,2'-stilbenesulfonic acid; DNDS, 4,4'-dinitro-2,2'-stilbenesulfonic acid; PCMBS, *p*-chloromercuriphenylsulfonic acid; DTDP, 4,4'-dithiodipyridine.

0005-2736/83/$03.00 © 1983 Elsevier Science Publishers B.V.

structural properties. Minor modifications, in particular of membrane proteins, may greatly perturb the mutual interactions of membrane elements and induce leaks which may be irreconcilable with cell viability. This report describes the formation and the properties of leaks with the characteristics of aqueous pores in human erythrocyte membranes upon cross-linking of spectrin.

Spectrin is the major constituent of the 'skeleton' formed by extrinsic membrane proteins at the cytoplasmic interface of the erythrocyte membrane [3–5]. This skeleton is connected to the hydrophobic, barrier-forming domain of lipids and intrinsic membrane proteins by protein-protein and protein-lipid interactions [5]. Its major purpose seems to be mechanical stabilization of the membrane [6,7]. In addition, the skeleton has been claimed to play a role in the maintenance of the transverse asymmetry and the suppression of the transverse mobility (flip-flop) of the membrane phospholipids [5]. Oxidation of membrane SH-groups to disulfides by diamide (diazine dicarboxylic acid-bis (dimethylamide)) or tetrathionate ($Na_2S_4O_6$), which goes along with cross-linking of spectrin monomers into oligomers [8–11], abolishes the predominant localization of the anionic amino-phospholipids at the inner layer of the membrane [12], presumably due to an enhancement of their transverse mobility. This enhancement could be demonstrated directly for phosphatidylcholine [13] as well as for a number of lysophospholipids [11,14].

In this report it will be shown that SH-group oxidation by diamide also induces a considerable leak permeability to nonelectrolytes and ions. The relationship between this leak permeability and the enhanced transverse mobility of phospholipids may provide some insight into the underlying perturbation of membrane structure by diamide, but will also be instructive with respect to natural leaks in unperturbed cell membranes. Preliminary reports have been given elsewhere [15,16].

## Materials and Methods

*1. Modification of SH-groups*

*(a) Oxidation.* Oxidation of membrane SH-groups was carried out in principle as described previously [8,12]. Washed erythrocytes were suspended at a hematocrit of 10% in a medium containing 100 mM KCl/50 mM NaCl/12.5 mM $Na_2HPO_4$-$NaH_2PO_4$/44 mM sucrose (pH 8.0, medium A). Part of the suspension served as control, part was exposed to diamide (diazene dicarboxylic acid-bis(dimethylamide), Calbiochem), or tetrathionate ($Na_2S_4O_6$), Fluka, Neu-Ulm, F.R.G.) or 4, 4'-dithiodipyridine (Serva, Heidelberg, F.R.G.) for time periods and at concentrations given in the results. After this treatment the samples were centrifuged; the cells were washed four times with medium A at pH 7.4 and immediately used for measurements of permeability. When short periods of exposure to diamide or other SH-reagents were necessary, the treatments were stopped by addition of a permeable (mercaptoethanol) or an impermeable (GSH) thiol, the former being used to quench alkylating SH-reagents, the latter for oxidative agents, in particular diamide. The permeability of diamide is so high [17] that its transmembrane diffusion will not significantly delay its reaction with GSH.

*(b) Other modifications.* Treatments of cells with alkylating SH-reagents (iodoacetate, iodoacetamide, *N*-ethylmaleimide) were carried out in a similar fashion at pH 8.0 including the subsequent stopping and washing procedure. Between consecutive treatments of cells with alkylating and oxidative reagents the cells were washed three times with medium A at pH 8.0. The reversibility of SH-oxidation was tested by treating the cells with dithioerythritol (Sigma, Munich) either during the preloading for tracer efflux measurements, or immediately after exposure to diamide.

*2. Modifications of the lipid domain*

Cells depleted of or enriched with cholesterol were obtained as described previously [18] by 48 h exposure to lipid vesicles prepared from DL-α-dipalmitoylphosphatidylcholine (Sigma, Munich No. P 6769) 1 mg/ml, with or without addition of 1.5 mg/ml cholesterol. Effects of detergents were tested by adding Brij 96 (polyoxyethylene-10-oleyl ether (Brij 96), Sigma, Munich) to the efflux media.

*3. Measurements of permeability*

Permeabilities of cells pretreated with SH-reagents were assessed by measuring tracer efflux from preloaded cells under selfexchange or net

flux conditions as described previously [19]. Briefly, cells were first loaded at a hematocrit of 3% with the unlabelled form of the nonelectrolyte or the anion to be tested in a medium containing, besides the test permeant at concentrations given in the results, 100 mM KCl/36 mM NaCl/6.5 mM $Na_2HPO_4$-$NaH_2PO_4$/44 mM sucrose (medium B). Test permeants were erythritol, L- and D-arabinose, L-glucose, sucrose, mannitol, acetate, glycolate, L-lactate and chloride, obtained from commercial sources, as well as *S*-acetamidothioglycol and *S*-acetamidothioglycerol. These two model nonelectrolytes were synthesized from iodoacetamide and mercaptoethanol or thioglycerol, respectively, by procedures to be described in detail elsewhere (Deuticke, B, Wilbers, K.H. and Schwisters, K., to be published). After equilibration of the cells with the unlabelled test permeants, the hematocrit was raised to 40% by removing part of the suspension medium. The cells were loaded with labelled test solutes, which were obtained from commercial sources (Amersham Buchler) except for *S*-[1-$^{14}$C]acetamidothioglycol or -thioglycerol, which were synthesized in analogy to their nonradioactive congeners.

Tracer efflux was initiated by resuspending tracer-loaded cells at a hematocrit of 5% in tracer-free medium. Rate coefficients $k$ of tracer exchange were derived by linear regression analysis from the slope of a plot of log $((cpm_\infty - cpm_t)/(cpm_\infty - cpm_0))$ versus time, subscripts 0, $t$ and $\infty$ referring to radioactivities (counts per min per ml) in the supernatant at the beginning, at any time, and after attainment of equilibrium. Permeabilities were calculated from rate coefficients according to the equation

$$P(\mathrm{cm \cdot s^{-1}}) = k(\mathrm{min^{-1}}) \cdot \frac{0.97}{60} \cdot \frac{V_{aq}}{A}$$

where 0.97 accounts for the compartment sizes under the conditions of our experiments, $V_{aq}$ = aqueous space of the erythrocyte (60 $\mu m^3$), $A$ = surface area of the cell (142 $\mu m^2$). Fluxes of permeants which use specific transfer systems in addition to 'unspecific' pathways were measured in the presence of appropriate inhibitors of the specific pathway: DIDS (a kind gift of Professor F.K. Schnell, Regensburg), DNDS (K and K), cytochalasin B (Sigma, Munich), PCMBS (Sigma, Munich).

## Results and Interpretation

*Formation of membrane leaks after SH-group oxidation*

Treatment of human erythrocytes with diamide (5 mM, hematocrit 10%, 45 min, 37°C, pH 8.0) remarkably enhances the rate of exit of a number of nonelectrolytes (Fig. 1). Under the conditions of our controls these test solutes permeate only by simple passive diffusion (presumably via the lipid domain of the membrane), since specific pathways were blocked by adequate inhibitors (cytochalasin B [20] for the glucose carrier transporting *m*-erythritol [21] and L-arabinose; $Cu^{2+}$ as an inhibitor of the glycerol transport system which accepts *S*-acetamidothioglycerol (Deuticke, B., unpublished data)). No hemolysis occurred during the efflux period, excluding cell destruction as a mechanism of the enhanced released. Efflux kinetics were linear for at least two half-times, suggesting a homogeneous distribution of the induced leak pathways over the cell population.

Effects similar to those of diamide were also observed after treatment of the cells with either tetrathionate or 4,4'-dithiodipyridine, known to

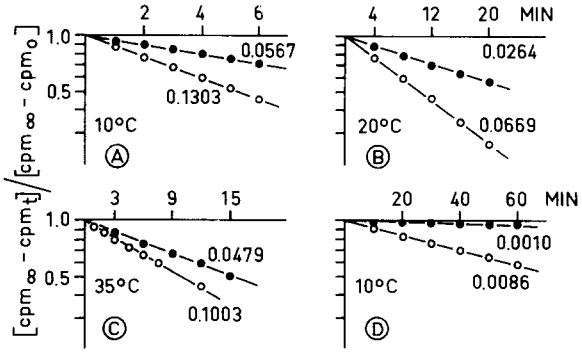


Fig. 1. Time course of the exit of labelled nonelectrolytes from normal (●) and diamide-treated (○) human erythrocytes. Cells were treated with diamide (5 mM, pH 8.0, 45 min). Fluxes measured at the temperatures indicated as described in the Methods. (A) *S*-acetamidothioglycol, (B) *S*-acetamidothioglycerol, (C) *m*-erythritol (in presence of cytochalasin B 10 $\mu$M), (D) L-arabinose (in presence of cytochalasin B 10 $\mu$M). Numbers on the graphs are the rate coefficients ($min^{-1}$), calculated from the slope of the linear regressions.

TABLE I

NONELECTROLYTE PERMEABILITIES OF HUMAN ERYTHROCYTES PRETREATED AT 37°C WITH OXIDIZING AND ALKYLATING SH-REAGENTS

Experimental details as given in the Methods. Numbers of experiments in brackets. Cyt., cytochalasin.

| | Rate coefficient of equilibrium exchange ($min^{-1}$) | |
|---|---|---|
| | $k_{SAM\text{-}thioglycol}$ (0°C) | $k_{Erythritol}$ (+10 μM Cyt. B) (35°C) |
| Control | 0.0610 ± 0.0076 (14) | 0.0448 ± 0.0044 (5) |
| Diamide 5 mM, 45 min | 0.1243 ± 0.0193 (14) | 0.1031 ± 0.0131 (5) |
| Tetrathionate 20 mM, 60 min | 0.1018 ± 0.0123 (4) | 0.0757 ± 0.0118 (5) |
| 4,4'-Dithiodipyridine 3.6 mM, 60 min | 0.1303 ± 0.0090 (4) | 0.0996 ± 0.0200 (4) |
| *N*-Ethylmaleimide 5 mM, 45 min | 0.0676 (1) | 0.0518 (1) |
| Iodoacetate 10 mM, 15 min | 0.0658 (1) | 0.0471 (1) |
| Iodoacetamide 2 mM, 45 min | 0.0647 (1) | 0.0478 (1) |

produce disulfide bonds in proteins [22] (Table I). In contrast, monofunctional, alkylating SH-reagents which either block a considerable fraction of membrane SH-groups [23,24] and/or diminish the cellular concentration of GSH [25] have at best a minor effect on membrane permeability (Table I) under comparable conditions. From this finding one may conclude that formation of peptide disulfide bonds is required for the effect. Neither a mere blockage of SH-groups nor the formation of mixed disulfides between membrane SH-groups and GSH, known to occur in diamide-treated erythrocytes [24], leads to changes of permeability. Specificity of the permeability changes is further suggested by observations compiled in Table II. The effect is reversible to a large extent when cells are exposed to dithioerythritol subsequent to treatment with diamide. Diamide-induced disulfide bonds are reduced [8] and spectrin oligomers reconverted into monomers [8] under these conditions. Reversibility thus provides indications for an involvement of spectrin in the enhancement of permeability. Further evidence in favour of this view comes from the observation that pretreatment of erythrocytes with *N*-ethylmaleimide almost completely suppresses the effect of a subsequent exposure to diamide and to tetrathionate (Table II). Under the conditions of this pretreatment *N*-ethylmaleimide reacts predominantly with spectrin [12]. The sensitivity of the membrane to this suppressive effect of *N*-ethylmaleimide is remarkably high: 0.4 mM (at a hematocrit of 10%) for 10–20 s suffice to induce full blockage (Fig. 2). Other alkylating SH-reagents (iodoacetate, iodoacetamide) are completely ineffective in this respect. Interestingly, the enhancement of permeability induced by DTDP is not suppressed by *N*-ethylmaleimide, suggesting a somewhat different mode of action of this reagent.

*2. Characteristics of leak formation by SH-group oxidation*

In view of the specificity of the enhancement of membrane permeability by diamide details of the phenomenon were studied. The increase of nonelectrolyte permeability depends on the concentration of diamide during pretreatment in a saturating fashion (Fig. 3A). Half-maximal enhancement is obtained at 1.3 mM (45 min exposure time). The maximum reached eventually depends on the exposure time. A saturating concentration dependence was also observed for the effect of tetrathionate (data not shown). At a constant concentration of diamide nonelectrolyte permeability rises linearily with the time of pretreatment up to al least 100–120 min, a surprising observation in view of the time course of oxidation of membrane SH-groups by diamide (dotted line in Fig. 3B), which proceeds nonlinearily as expected (cf. Ref. 12) and makes only little further progress at a time at which the permeability still rises progressively. In spite of these different time courses the increase

TABLE II

CHARACTERISTICS OF THE EFFECT OF DIAMIDE AND 4,4'-DITHIODIPYRIDINE (DTDP) ON NONELECTROLYTE PERMEABILITY OF HUMAN ERYTHROCYTES

For testing suppressive effects, cells were pretreated with *N*-ethylmaleimide (NEM) or iodoacetamide (pH 8, 37°C), reversibility was established by exposure to dithiothreitol after diamide treatment and washing of cells. Numbers of experiments in brackets. Cyt. cytochalasin.

| | Rate coefficient of equilibrium exchange ($min^{-1}$) | |
|---|---|---|
| | $k_{SAM\text{-}thioglycol}$ 0°C) | $k_{Erythritol}$ (+ 10 μM Cyt. B) (35°C) |
| Control | 0.0630 ± 0.0051 (4) | 0.0423 (2) |
| Diamide 5 mM, 45 min | 0.1037 ± 0.0064 (4) | 0.0833 (2) |
| NEM 0.4 mM, 15 min, then diamide 5 mM, 45 min | 0.0657 ± 0.0046 (4) | 0.0486 (2) |
| Iodoacetamide 2 mM, 15 min, then diamide 5 mM, 45 min | 0.1054 (2) | n.d. |
| Tetrathionate, 20 mM, 60 min | n.d. | 0.0726 ± 0.0116 (3) |
| NEM 0.4 mM, 15 min, then tetrathionate 20 mM, 60 min | n.d. | 0.0510 ± 0.0033 (3) |
| DTDP 5 mM, 60 min | 0.2095 ± 0.0050 (3) | n.d. |
| NEM 0.4 mM, 15 min then DTDP 5 mM, 60 min | 0.2356 ± 0.0148 (3) | n.d. |
| Diamide 5 mM, 45 min, then dithioerythritol 10 mM, 75 min | 0.0763 ± 0.0146 (4) | 0.0594 (2) |

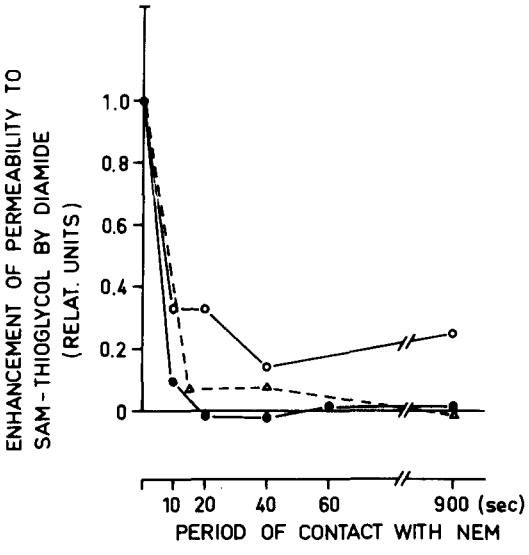


Fig. 2. Suppression of the effect of diamide on *S*-acetamidothioglycol permeability by pretreatment of erythrocytes with *N*-ethylmaleimide. Cells were first treated with iodoacetate (1.3 μM, pH 8.0, 15 min) to block glutathione, then exposed (pH 8.0, 37°C, hematocrit 10%) to *N*-ethylmaleimide (NEM) (Δ, 0.6 mM; ●, 0.4 mM; ○, 0.2 mM) for the periods indicated. The exposure was terminated by addition of glutathione (1 mM) to the suspension to quench unreacted *N*-ethylmaleimide. Subsequently, cells were washed and exposed to diamide (45 min, pH 8.0, 37°C). Fluxes of *S*-acetamidothioglycol measured at 0°C as described in the Methods. 1.0 on the ordinate refers to the increase of the permeability ($\Delta P$) in cells only exposed to diamide (relative units). SAM, *S*-acetamido.

of permeability must be closely related to the oxidative effects of diamide. Permeability does not increase further during a prolonged (up to 3 h) incubation of diamide-treated cells in the absence of the agent (data not shown). This indicates that

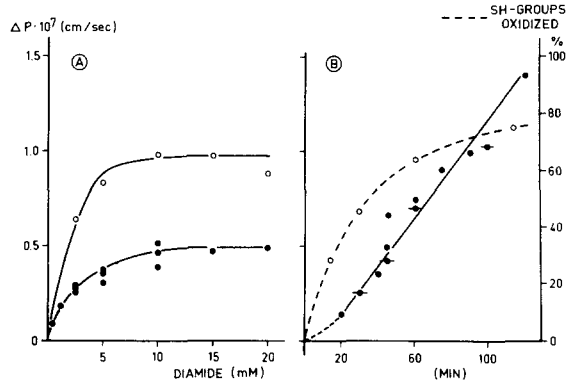


Fig. 3. Concentration-and time-dependence of the effect of diamide on *S*-acetamidothioglycol permeability at 0°C. The diamide effect is defined as the increase of permeability ($\Delta P$) on top of the control permeability ($0.44 \cdot 10^{-7}$ $cm \cdot s^{-1}$) of *S*-acetamidothioglycol at 0°C. (A) Cells treated with diamide (pH 8.0, 37°C) for 45 (●) or 100 (○) min. (B) Cells treated with diamide 5 mM (●) or 40 mM (-●-) at 37°C, hematocrit 30%, after preloading with labelled and unlabelled *S*-acetamidothioglycol. Exposure to diamide was stopped by addition of glutathione (15 mM). Dotted line: percent of oxidized SH-groups determined as described elsewhere [8,12]. Normal content of SH-groups 80–90 $nmol \cdot mg^{-1}$ membrane protein.

the presence of the oxidant is an essential prerequisite for the increase of permeability.

In line with the involvement of dissociable SH-groups in the effect of diamide the effect on permeability increases progressively with increasing pH during treatment (data not shown). The 'titration curve' for permeability suggests an apparent p*K*-value of the SH-groups reacting with diamide above a pH value of 8.5, since no inflection point of the curve was observed up to this pH.

A linear time dependence combined with a saturating concentration dependence for diamide's effect on permeability indicates that it is not the availability of the oxidant which limits the rate of the enhancement of permeability. This is also very unlikely in the light of the high membrane permeability to diamide [17]. Moreover, the difference in the time courses between the overall SH-group oxidation and the enhancement of permeability excludes the rate of overall SH-oxidation as the rate limiting factor.

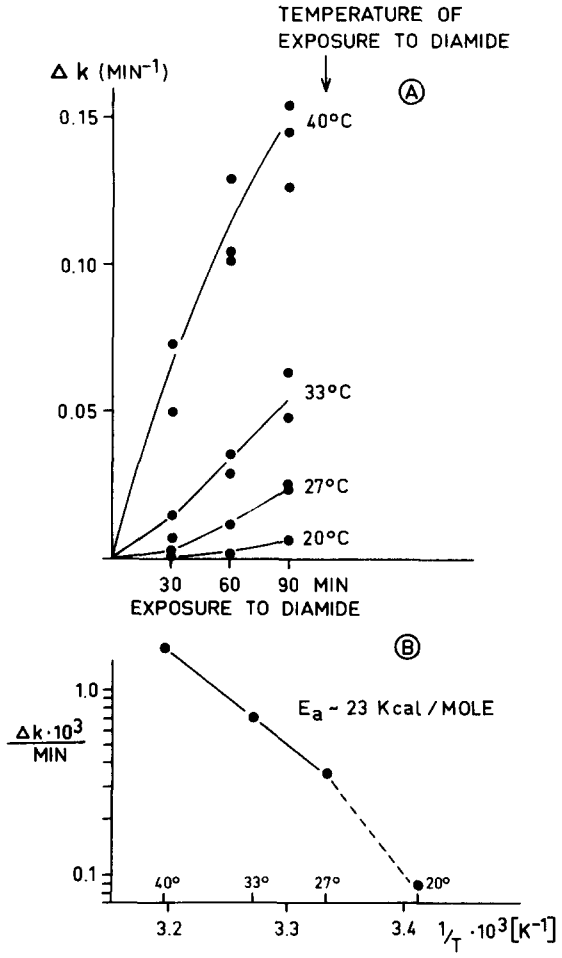


Fig. 4. (A) Time course of the diamide-induced enhancement of *S*-acetamidothioglycol permeability at different temperatures. Cells were exposed to diamide (5 mM) at the temperatures indicated after preloading with *S*-acetamidothioglycol. The reaction was stopped by addition of glutathione (15 mM) and fluxes measured at 0°C immediately thereafter. (B) Arrhenius diagram derived from the slope for the increase of permeability with time.

A crucial role of steps preceding or following the step of disulfide formation is also suggested by the temperature dependence for the development of an increased permeability (not to be mixed up with the temperature dependence of the permeability induced by diamide). As shown in Fig. 4A, diamide treatment at 20°C (or below) has almost no influence on permeability. SH-group oxidation is decreased to some extent at 20°C (40% oxidized after 60 min instead of 65% at 37°C). Crosslinking of spectrin is almost completely suppressed (data not shown). At higher temperatures the rate at which permeability increases during diamide exposure is markedly accelerated by increasing temperatures. The slopes can be evaluated in an Arrhenius diagram (Fig. 4B) which reveals an apparent activation energy of about 23 kcal/mol between 27 and 40°C. This value is considerably higher than the activation energy of the oxidation of SH-groups in low-molecular thiols or soluble proteins (Haest, C.W.M., unpublished data).

*3. Characteristics of the pathway induced by diamide*

An enhancement of passive membrane permeability to nonelectrolytes could in principle result from changes of the properties of the lipid domain and would then be comparable, e.g., to the consequences of cholesterol depletion or enrichment [2], or the insertion of lipid analogues [26] or treatment with short aliphatic alcohols [2]. On the other hand, small polar nonelectrolytes like those studied here would be able to penetrate via aqueous pores. The increase of the transbilayer mobility of phospholipids in diamide-treated cells [8,13,14] clearly suggests an alteration of the lipid domain. However, the mechanism of transbilayer motions of phospholipids not being known, the formation of aqueous pores cannot be dismissed. Such pores should be permeable to charged permeants of ade-

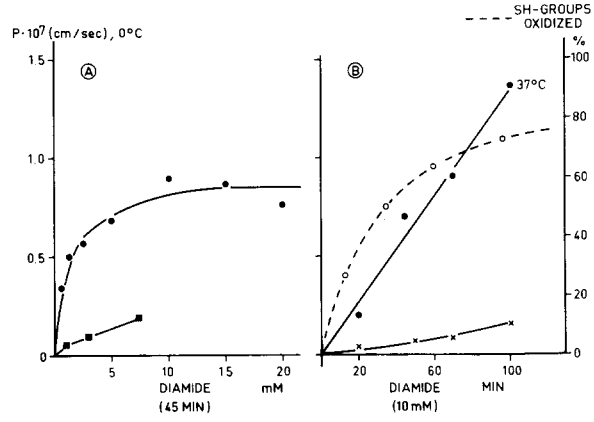


Fig. 5. Concentration- and time-dependent changes of chloride leak permeability of human erythrocytes after pretreatment with diamide. Cells suspended in medium A (hematocrit 30%) were loaded with $^{36}Cl^-$ (10 min, 37°C, pH 8.0), then exposed to DIDS (10 $\mu$M, 30 min, 37°C) and subsequently treated with diamide (45 min, 37°C). This treatment was stopped by addition of glutathione (15 mM). Cells were centrifuged and $^{36}Cl^-$ efflux into medium A (+ 10 $\mu$M DIDS) measured immediately at 0°C. (A) Concentration dependence. ●, Cells treated with diamide; ■, cells treated with diamide, then exposed to dithioerythritol (7.5 mM) for 45 min at pH 8.0, 37°C. (B) Time dependence. ●, cells treated with diamide; ×, cells pretreated with 0.4 mM *N*-ethylmaleimide, 15 min, pH 8.0, 37°C prior to diamide exposure. ○, SH-group oxidation.

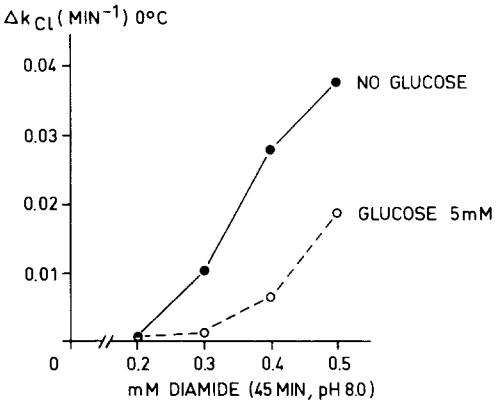


Fig. 6. Protective action of glucose against the enhancement of $Cl^-$ leak permeability by diamide. Experimental procedure as in Fig. 5A, except that glucose (5 mM) was added to one of two samples.

quate size. We therefore studied passive leak permeabilities to small ions in diamide-treated cells by means of chloride tracer fluxes. $Cl^-$ leak permeability was defined as the component of $Cl^-$ equilibrium exchange insensitive to extensive pretreatment of cells with DIDS, a potent irreversible inhibitor of mediated $Cl^-$ transport [2]. This leak permeability was $3 \cdot 10^{-10}$ cm · $s^{-1}$ at 0°C pH 7.4, in our hands. Diamide pretreatment indeed produced a dramatic enhancement of chloride permeability (Fig. 5). An increase by almost three orders of magnitude was observed after 100 min pretreatment. This enhancement might in principle result from a loss of the well documented [27] specific inhibition of $Cl^-$ transfer by DIDS. The inhibitory potency of stilbenedisulfonate inhibitors, however, is fully preserved in diamide-treated cells (Deuticke, B., unpublished data). $K^+$ permeability, estimated by following net exits of $K^+$ from diamide-treated cells into choline chloride media with an ion-sensitive glass electrode, was found to be enhanced to the same extent as $Cl^-$ permeability (Deuticke, B. and Schwister, K., unpublished data).

The increase of ion permeability produced by diamide exhibits all characteristics observed for the enhancement of nonelectrolyte permeability, including suppression by *N*-ethylmaleimide and reversibility upon cleavage of disulfide bridges by dithioerythritol (Fig. 5). The relationship between the reversible oxidation of SH-groups and the enhancement of chloride permeability could be demonstrated conspicuously by a protective action of the presence of glucose (Fig. 6). The glucose-induced retardation of the increase of chloride permeability is accompanied by a retarded disappearance of membrane SH-groups. After 45 min treatment with 0.5 mM diamide SH-groups had decreased by 17% in the absence of glucose and by 9% in its presence. Cross-linking was also delayed in the presence of glucose (Haest, C.W.M., unpublished data). Glycolytic metabolism obviously antagonizes the cross-linking of spectrin by providing reducing equivalents for a continuous recleavage of disulfide bonds by NADPH or GSH (see also Refs. 9, 24, 28 and 29).

The diamide-induced enhancement of ion permeability is a strong argument against a mechanism involving mere changes of the barrier properties of the lipid domain, e.g. a 'fluidization'. Only a very polar pathway, equivalent to an aqueous pore, will allow for the rapid permeation of small ions. Such a pathway should discriminate per-

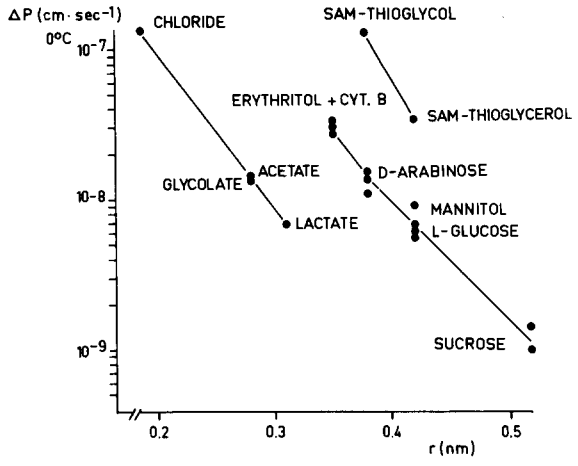


Fig. 7. Relationship between the diamide-induced permeabilities of various test solutes and their radii. For studies involving sucrose, L-glucose, mannitol and D-arabinose cells were suspended in medium A (pH 8.0, 37°C, hematocrit 10%) containing the unlabelled test solutes. Diamide (10 mM) was added for 120 min. The reaction was stopped by adding 15 mM glutathione, and the cells loaded with $^{14}$C-labelled test solute for 60 min at 37°C. The suspension was then cooled to 0°C, the cells spun down and tracer efflux into medium A, containing only the unlabelled solute, measured at 0°C. Cytochalasin B (10 $\mu$M) was added in experiments with *m*-erythritol.

For studies involving other nonelectrolytes cells were treated and fluxes measured at 0°C as described in the Methods. Fluxes of $Cl^-$ measured as described in Fig. 5. Fluxes of organic anions determined in presence of 0.3 mM DNDS and 0.2 mM PCMBS in order to block anion movements via specific transport systems [2,27]. The radii of carbon compounds are viscometric radii, taken for the polyhydroxy compounds from Ref. 92. Radii for the organic anions and for the *S*-acetamido-substituted thiols were supposed to be equal to the radii of polyhydroxy compounds of the same number of C-atoms (S-atoms in the *S*-acetamido derivatives set alike to a C-atom in this function). Hydrated radii for $Cl^-$ taken from Ref. 93 (Table 14.4, p. 271). SAM, *S*-acetamido.

meants according to their molecular size. The diamide-induced permeabilities of a number of polar nonelectrolytes and ions, plotted in Fig. 7 against their radii, bear out this expectation. Since erythrocytes are factually impermeable ($P < 10^{-10}$ cm · s$^{-1}$) to mannitol, D-arabinose, L-glucose and sucrose (Ref. 30, and Deuticke, B., unpublished data), control values could not be established in these experiments. Cells were loaded with the test solutes via the leaks induced by diamide. As is evident from the graph, diamide-treated cells become permeable to polyhydroxy-compounds up to the size of sucrose. These observations suggest the formation of pores with equivalent radii of at least 0.5–0.6 nm. Interestingly, the data points for the test compounds studied could not be fitted by one single curve. The permeability versus size relationship for monovalent anions indicates a somewhat lower pore radius, acetamido-substituted polyols, which are less hydrophilic than the unsubstituted polyhydroxy compounds, suggest larger radii. The diamide-induced pathway obviously dis-

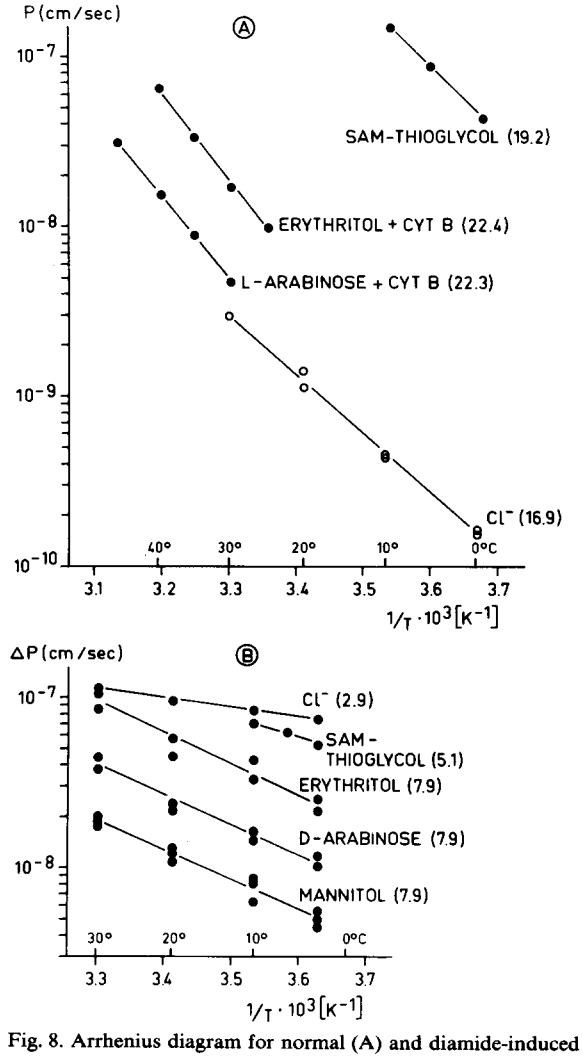


Fig. 8. Arrhenius diagram for normal (A) and diamide-induced (B) leak fluxes in human erythrocytes. Cells were treated with diamide and fluxes measured as described in the Methods and in Figs. 1, 5 and 7. Leak fluxes of $Cl^-$ in normal cells defined as the DIDS-insentive flux. Numbers in brackets are activation energies (kcal/mol). SAM, *S*-acetamido.

criminates permeants not only by virtue of their size but also by their polarity. Nevertheless, the pathway has to be regarded as an aqueous pore, as becomes unequivocally clear from the apparent activation energies of the diamide-induced permeability. In the native membrane the passive, non-mediated transfer of nonelectrolytes such as *S*-acetamidothioglycol, polyols, monosaccharides and of $Cl^-$ has a characteristic activation energy of 16–22 kcal/mol (Fig. 8A), usually regarded as the consequence of the removal of hydration water in the course of membrane permeation [31]. The diamide-induced permeability of the same solutes is much less temperature dependent (Fig. 8B). Activation energies range from 2.5 to 8 kcal/mol, very close to the activation energy for free diffusion of polar nonelectrolytes or ions in an aqueous environment, not requiring dehydration.

*4. Relevance of membrane lipid for leak formation*

The polarity of the permeants, the activation energy of the fluxes, and the selectivity of the pathway induced by diamide clearly indicate that aqueous pathways have been formed. On the other hand, the lipid phase is also involved in the process. The enhanced transbilayer mobility of phospholipids [8,13,14] and the dependence of selectivity on solute polarity (Fig. 7) provide inferential evidence for this idea. More direct proof comes from the observation that perturbation of the membrane lipid domain modifies the effect of diamide. According to Table III cholesterol depletion (by about 50%) enhances the normal and the diamide-induced permeability to almost the same extent. Cholesterol enrichment (by about 90%) has no effect on the diamide-induced pathway but reduces the normal permeability. In these experiments membrane cholesterol content was altered before the diamide treatment. Thus, it cannot be decided whether the process of leak formation per se or properties of the leak were altered. This uncertainty does not prevail when membranes are modified only after the diamide exposure, as was the case with hexanol and Brij 96. These compounds, which will insert into the lipid domain of the membrane, stimulated the normal as well the diamide-induced pathway (Table III).

A final feature of the diamide-induced leak which may be related to membrane lipids concerns a marked species difference in diamide sensitivity. Ox erythrocytes do not develop a leak when exposed to diamide although their membrane SH-groups are oxidized and spectrin is cross-linked as in the human red cell (data not shown). Flip rates of lysophospholipids are also insensitive to diamide in ox erythrocytes (Dressler, V. and Haest, C.W.M., data not shown). The high content of sphingomeylin in bovine erythrocyte membranes, characteristic for these cells [2], must certainly be considered as a reason for the diamide insensitivity.

TABLE III

INFLUENCE OF MODIFICATIONS OF THE LIPID DOMAIN ON THE NORMAL AND THE DIAMIDE-INDUCED PERMEABILITY OF THE ERYTHROCYTE MEMBRANE

Data are expressed relative to the rate coefficient of the respective controls ( = 1.0). Membrane levels of cholesterol were changed prior to diamide treatment, hexanol and Brij 96 added to the efflux media. Mean values from three or two experiments. Erythritol fluxes measured in presence of 10 μM cytochalasin B. SAM, *S*-acetamido.

| | Normal pathway | | Diamide-induced pathway | |
|---|---|---|---|---|
| | SAM-thioglycol (0°C) | Erythritol (35°C) | SAM-thioglycol (0°C) | Erythritol (35°C) |
| Cholesterol-depleted | 1.63 | 1.20 | 1.63 | 1.29 |
| Control | 1.0 | 1.0 | 1.0 | 1.0 |
| Cholesterol-enriched | 0.84 | 0.81 | 1.03 | 0.96 |
| Hexanol 10 mM | 1.33 | n.d. | 1.96 | n.d. |
| Brij 96 60 μM | 1.40 | n.d. | 2.13 | n.d. |

## Discussion

*1. Membrane modification by SH-reagents*

Modification of membrane SH-groups by alkylating reagents and mercurials has long been known to increase erythrocyte membrane permeability to cations [32,33]. Consequences of SH-group oxidation have only been studied to a limited extent under the aspect of membrane permeability [34–36], although 'oxidative stress' in a more general sense has often tacitly been assumed to induce hemolysis via a disturbance of the barrier properties of the erythrocyte membrane [37–40].

Effects on erythrocytes of the two SH-oxidizing agents used in our study are known from earlier studies. Diamide was introduced by Kosower as a highly permeable mild oxidant of glutathione not acting via radical formation and not oxidizing human hemoglobin [41,42]. Moreover, diamide oxidizes membrane SH-groups [8], forms mixed disulfides of glutathione with hemoglobin [43] and membrane thiols [24], and inhibits cytoplasmic and membrane-bound enzymes of the erythrocyte (Refs. 44, 45, and Haest, C.W.M., unpublished data).

Tetrathionate (see Ref. 22 for additional information), which enters the erythrocyte slowly via the anion exchange system [46] inhibits glycolytic enzymes by oxidation of SH-groups [47], but also stimulates red cell glycolysis [48] and produces reversible oxidation of GSH [49]. Disulfide formation by both agents goes along, in native cells, with pronounced intermolecular cross-linking of spectrin [8,9,11]. Other membrane proteins predominantly form intramolecular disulfide bonds and mixed disulfides, e.g. with GSH [24]. Small amounts of peptides from other fractions are found in the high molecular weight complexes of spectrin after more extensive treatments [9,11]. An enhancement of passive leak permeability to cations in red blood cells by the two reagents has already been reported [34,50,51]. Our present study clearly shows a general disturbance of the barrier properties of the membrane.

*2. Spectrin cross-linking and aggregation as a cause of leak formation?*

A possible link between the oxidation of spectrin by diamide and tetrathionate and the disturbance of membrane barrier properties is provided by observations indicating that the cross-linking process is paralleled by aggregation of membrane skeletal proteins and of the membrane-intercalated particles as demonstrated by freeze-fracture electron microscopy [11,52]. Intermolecular cross-linking or aggregation readily account for two other effects of both reagents, namely a pronounced membrane 'stiffening' [7] and a decrease of lateral mobility of intrinsic membrane proteins [10]. The size of the oligomers of spectrin linked by disulfide bonds is not yet defined clearly. A minimal molecular weight of $1 \cdot 10^6$, indicating tetramers, has been reported [12]. It remains to be elucidated therefore, whether the visible aggregates are linked covalently, which process triggers and which forces drive the aggregation.

Cross-linking of membrane proteins and aggregation of membrane skeletal elements as well as of membrane spanning proteins render membranes leaky under other conditions, too. Most conspicuously, Bjerrum [30] has shown that titration of resealed erythrocyte ghosts to the isoelectric point of spectrin (pH approx. 4.9) goes along with a reversible aggregation of membrane proteins and a reversible formation of membrane leaks with the properties of aqueous pores ($r > 0.5$ nm), pervious to ions and nonelectrolytes. Furthermore, photooxidation of the erythrocyte membrane in the presence of porphyrins [37,53] induces cross-linking of membrane proteins [54,55], particle aggregation [56] as well as an increase of nonelectrolyte and ion permeabilities leading to photohemolysis [38–40,57]. The leak pathway is not well characterized, but may be an aqueous pore in view of the low activation energy of photo-induced $K^+$ permeability of 2.5 kcal/mol [39] and its permselectivity for hydrophilic nonelectrolytes reconcilable with an equivalent radius $> 2$ nm [57]. The primary mechanism underlying photodynamic membrane modification is most certainly not SH-group oxidation [58]. The patterns of protein cross-linking are different [54,55] and membrane lipids, which are not affected by diamide [34], are also modified [37]. A further example of leaks induced by cross-linking may be cells treated with $Cu^{2+}$ ions undergoing oxidative hemolysis [59].

While leaks with the properties of aqueous pores thus are formed in parallel with different types of

protein aggregation in the erythrocyte membrane, cross-linking does not produce pores inevitably. Cross-linking of spectrin by γ-glutamyl-ε-lysyl bonds following $Ca^{2+}$-activation of transglutaminase [60], for instance, does not affect membrane permeability (Deuticke, B., unpublished data). On the other hand, leaks resembling aqueous pores can be induced in erythrocyte membranes without spectrin cross-linking by dielectric breakdown (Refs. 61, 62, and Schwister, K. and Deuticke, B., to be published).

Thus, there is obviously not a simple relationship between cross-linking of membrane skeletal proteins and leak formation. It might therefore be asked, whether it is really the intermolecular cross-linking of spectrin and not some intramolecular disulfide-bond formation, or thiol blocking, that is involved. Thiol blocking can be excluded on the basis of our finding that extensive treatment with *N*-ethylmaleimide does not produce leaks comparable to those induced by diamide (cf. Table I). Three arguments support intermolecular cross-linking as the basic event: (1) Pretreatment of cells with low concentrations of *N*-ethylmaleimide suppresses the formation of leaks (Table II) and reduces the extent of spectrin cross-linking [12], but does only slightly diminish the overall oxidation of SH-groups (Haest, C.W.M., unpublished data). *N*-ethylmaleimide reacts preferably with spectrin (three molecules per spectrin monomer) under these conditions [12]. (2) Treatment with diamide at low temperatures diminishes the formation of leaks (Fig. 6) and suppresses intermolecular crosslinking. 3) Oxidation, by $Cu^{2+}$-*o*-phenanthroline, of SH-groups in spectrin-free inside-out vesicles does not affect action permeability [63]. Dimerization of band 3 protein, which occurs under these conditions [64] can therefore be excluded as a reason for leak formation in erythrocytes. This view is also supported by our finding that leak formation after diamide is not changed when band 3 protein is dimerized, besides spectrin cross-linking, in cells pretreated with iodoacetate. Iodoacetate pretreatment removes glutathione [49] which normally prevents band 3 dimerization by forming a mixed disulfide with band 3 protein [24].

On the other hand, the very different time courses of the overall SH-group oxidation and of leak formation (Figs. 3 and 5) indicate steps intermediate between the two events. One of these may be the process of protein aggregation, which has not yet been studied in its time dependence. Alternatively, it has to be considered that not cross-linking in general, but the slow oxidation of a small subpopulation of SH-groups finally produces the leaks. The high temperature dependence for the development of diamide's effect (Fig. 4) indicates that a considerable energy barrier has to be overcome in forming the leaks.

*3. Size and number of the induced leaks*

An important aspect of the induced leaks relates to the question of their size and number. Qualitatively, the data in Fig. 7 suggest the presence of pores with radii up to at least 0.5 nm. Radii derived from different classes of test solutes probably vary to some extent, less polar permeants indicating larger radii. This feature may reflect some sort of partition of permeants between the aqueous bulk phase and the interior of the pore, determined by nonpolar forces. Artificial systems with molecular sieving properties (swollen dextran gels) also discriminate solutes on the basis of hydrophobic interactions [65].

For a further, refined analysis it has to be considered that conceptually the induced permeability is proportional to the total area ($A_{tot}$) of the induced leaks. $A_{tot}$ for a single erythrocyte equals the product of $N_p$, the number of pores per cell, and $A_p$, the area of a single pore. Consequently $A_{tot} = N_p \cdot A_p = N_p \cdot \pi\, r_p^2$, where $r_p$ is the radius of a single cylindrical pore.

The total area obtainable from the experimental data (see below) is not equal to $A_{tot}$ but has a smaller value, since collisional sieving restricts diffusion of test solutes through the pore; yielding only apparent total areas of diffusion

$$A_{app} = N_p \cdot \pi \cdot (r_p - r_s)^2 \qquad (1)$$

where $r_s$ is the radius of the test solute [66].

According to Fick's law the flux $J$ through the sum of the pores induced in a single cell is $J = D_p \cdot A_{app} \cdot \Delta C / l_p$, where $D_p$ = diffusion coefficient within the pore, $l_p$ = pore length, and $\Delta C$ = transmembrane concentration difference driving the flux. On the other hand, the fluxes are also

describable by $J = P_{Ery} \cdot A_{Ery} \cdot \Delta C$, where $P_{Ery}$ is the permeability coefficient obtained in the usual way (see Methods). Hence

$$P_{Ery} \cdot A_{Ery} = \frac{D_p}{l_p} \cdot A_{app} \quad (2)$$

and

$$A_{app} = P_{Ery} \cdot A_{Ery} \cdot \frac{l_p}{D_p} \quad (3)$$

Values for $N_p$ and $r_p$ can be derived from Eqn. 1 when combined and solved for pairs of permeants with radii $a$ and $b$ [67]. One obtains

$$r_p = \frac{b\sqrt{{}^{a}A_{app}} - a\sqrt{{}^{b}A_{app}}}{\sqrt{{}^{a}A_{app}} - \sqrt{{}^{b}A_{app}}} \quad (4)$$

$$N_p = \frac{{}^{a}A_{app}}{\pi(r_p - r_a)^2} = \frac{{}^{b}A_{app}}{\pi(r_p - r_b)^2} \quad (5)$$

This approach has recently been proposed and successfully applied by Lieber and Steck [67,68] to define $N_p$ and $r_p$ for holes in osmotic ghosts. We used $A_{app}$ values calculated from the permeabilities and viscometric radii as given in Fig. 7. A value of 5 nm (i.e. of this thickness of the lipid bilayer) was chosen as a reasonable approximation of the pore length [69]. Diffusion coefficients inside the pore will equal maximally those in bulk water. A somewhat lower value may be more realistic for narrow pores. Diffusion coefficients within the gramicidin A channel (internal diameter about 0.4 nm) have recently been estimated to be about one order of magnitude lower than those in bulk solution [70]. This may provide a lower limit for the diffusion coefficients inside the diamide-induced pore.

As becomes evident from Table IV, different equivalent pore radii and pore numbers are obtained for different pairs of permeants. Pore radii decrease slightly with decreasing radii of the permeants, while pore numbers increase markedly, from about 1 per cell for sucrose to 17 for erythritol. Variation of the assumptions concerning $D_p$ or $l_p$, while not affecting the pore radii obtained, leads to different pore numbers, but does not alter the ratio between them. Faulty measurements of permeability are not very likely to account for this result, since permeabilities for erythritol, calculated by Eqn. 3 on the basis of the pore size and number for sucrose, were lower by a factor of 3 than the measured permeability of erythritol, far out of the range of experimental errors. More likely, the results reflect pore heterogeneity. This does not contradict the linear efflux kinetics referred to above, if heterogeneity concerns each cell to about the same extent and heterogeneity in the cell population is not involved. In view of these results it seems so far safe to assume that diamide treatment induces pores with radii up to about 0.65 nm but scattering around a somewhat lower mean (or mode) value. The number of pores per cell is most certainly larger than 1, probably even larger than 10 (or 100 when the diffusion coefficients within the pores are lower than those in bulk water). The diamide-induced leaks are thus very different from those present in osmotic ghosts, in which only a single, but very large ($r_p \leqslant 1.4$ nm) hole seems to be present [67,68].

The total area ($A_p \cdot N_p$) of the induced pores per cell is certainly very small. This is also documented by the observation that the water permeability of diamide-treated cells established by a simple technique [71], is unaltered (Osberghaus, U., unpublished observations). From the above

TABLE IV

RADII AND NUMBERS OF PORES INDUCED BY DIAMIDE

Values calculated from permeabilities of pairs of permeants as described in the text, using the viscometric radii from Fig. 7 and the following diffusion coefficients ($\times 10^5$ cm$^2 \cdot$s$^{-1}$): sucrose 0.27, L-glucose 0.31 (from Ref. 94, corrected to 0°C). D-Arabinose 0.35, erythritol 0.40. Coefficients for the latter two compounds where calculated from the square root of their molecular weight ($\sqrt{M}$) and the product $\sqrt{M} \cdot D$ for sucrose or glucose, assuming constancy of this product for the 4 solutes tested [95].

| Calculated from permeabilities for | Pore radius (nm) | Pores per cell |
|---|---|---|
| Sucrose, glucose | 0.63 | 1 |
| Glucose, arabinose | 0.52 | 4 |
| Arabinose, erythritol | 0.45 | 17 |

calculations water permeability would be expected to increase by about $10^{-6}$ cm · $s^{-1}$ in diamide-treated cells. This enhancement will not be detectable against the background of the normal water permeability of the erythrocyte of $3 \cdot 10^{-3}$ cm · $s^{-1}$ [71].

*4. Molecular basis of the induced leaks*

The molecular nature of the induced pores can only be considered in speculative terms. Conceptually, they may be situated (a) in the lipid domain, (b) at the interface between the lipids and intrinsic membrane-spanning proteins, or (c) within aggregates of proteins. An involvement of the lipid domain is clearly suggested by the enhancement of the transbilayer mobility of phospholipids [8,13,14] and fatty acids [11] in diamide-treated erythrocytes, although it is not yet established that the two events occur at the same sites. The sensitivity of the diamide-induced leak to changes of membrane cholesterol and to reagents perturbing the membrane lipid domain (Table III) provide additional evidence for the involvement of lipids.

The aggregation of skeletal proteins and of membrane-intercalated particles in diamide-treated cells [11,52] produce lipid areas free of membrane proteins and, possibly, lipid areas perturbed at their cytoplasmic surface by aggregates of extrinsic proteins. In either case, reorganization of phospholipids, e.g. local phase separations, may occur, giving rise to the formation of hydrophobic pores, characterized (Fig. 9) by an increase in lateral compressibility and thinning of the bilayer [72,73]. Alternatively, hydrophilic pores might arise [74]. Such pores would have to be envisaged as fluctuating structures, since stable hydrophilic pores in lipid bilayers require a diameter equal to the thickness of the membrane [75]. The pore radius would thus have to be greater than or equal to 2.5 nm, which is not compatible with our results. Moreover, non-bilayer phases intercalated into the original bilayer domain could be formed [76]. Such perturbed lipid structures, which also promote an enhanced transbilayer mobility of phospholipids [77], are known from studies on artificial systems to be much more permeable, even to ions, than 'normal' mixed bilayers containing cholesterol.

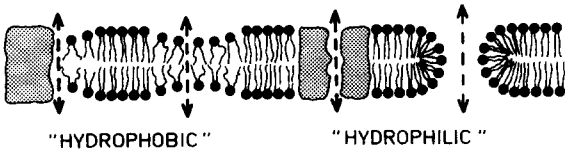


Fig. 9. Possible structures acting as aqueous pores in diamide-treated erythrocytes.

The interface between the lipid bilayer and membrane-spanning proteins is tightly sealed in normal biomembranes, although the rugged surface of the protein has to make contact with alkyl chains in this region. A lateral displacement of intrinsic membrane proteins into new environments may produce a 'mismatch' between lipids and peptide chains acting as an aqueous pore [78]. In model studies proteins inserted into lipid bilayers have been shown to induce considerable leak permeabilities [72–82], again in parallel with an increased transverse mobility of phospholipids [80,82,83].

Finally, aggregates of intrinsic proteins might form aqueous channels penetrating the lipid bilayer even though the walls of these channels would be constituted by rather hydrophobic protein domains. The polar residues which are present in the intra-bilayer domains of membrane proteins [84,85], however, are sufficient to reduce the activation barrier that has to be overcome by ions permeating the hydrophobic core of a normal lipid bilayer [86].

Oligomeric arrangements of exogenous and endogenous peptides are well established as channels in a number of instances: annular arrangements of proteins form junctions between adjacent cells [87], and control the permeation of material across the bacterial [88] and mitochondrial outer membrane [89], to name only a few.

The pores induced by diamide may be considered as a model for damages in the erythrocyte membrane induced by oxidative stress in vivo or in vitro. Furthermore, the leaks demonstrated here are a promising model for naturally occurring pores. The presence of aqueous pores, although of somewhat smaller diameter, has been claimed in the erythrocyte membrane for a long time [90,91], but their detailed properties could not yet be defined. Our studies provide a means to induce pores in the erythrocyte membrane without introducing foreign material. It will be interesting to

study further properties and the origin of these pores in more detail. Such studies are presently in progress.

## Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 160/C3). The authors wish to thank Mrs. D. Kamp for carrying out the SH-group determinations and Mr F.-J. Kaiser for preparing the graphs. The secretarial help of Mrs. H. Thomas is greatly appreciated.

## References

1 Beaugé, L. and Lew, V.L. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 38–52, Academic Press, London, New York, San Francisco
2 Deuticke, B. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 1–97
3 Gratzer, N.B. (1981) Biochem. J. 198, 1–8
4 Bennett, V. (1982) J. Supramol. Struct. Cell. Biochem. 18, 49–65
5 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352
6 Marchesi, V.T. (1979) J. Membrane Biol. 51, 101–131
7 Fischer, T.M., Haest, C.W.M., Stöhr, M., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 510, 270–282
8 Haest, C.W.M., Kamp, D., Plasa, G. and Deuticke, B. (1977) Biochim. Biophys. Acta 469, 226–230
9 Kosower, N.S., Kosower, E.M., Zipser, Y., Faltin, Z. and Shomrat, R. (1981) Biochim. Biophys. Acta 640, 748–759
10 Smith, D.K. and Palek, J. (1982) Nature 297, 424–425
11 Mohandas, N., Wyatt, J., Mel, S.F., Rossi, M.E. and Shohet, S.B. (1982) J. Biol. Chem. 257, 6537–6543
12 Haest, C.W.M., Plasa, G., Kamp, D. and Deuticke, B. (1978) Biochim. Biophys. Acta 509, 21–32
13 Franck, P.F.H., Roelofsen, B. and Op den Kamp, J.A.F. (1982) Biochim. Biophys. Acta 687, 105–108
14 Haest, C.W.M., Bergmann. W., Plasa, G. and Deuticke, B. (1980) Biophys. Struct. Mech. 6 (Suppl.) (Abstract) 98
15 Deuticke, B., Forst, B. and Poser, B. (1980) Biophys. Struct. Mech. 6 (Suppl.) (Abstract) 70
16 Deuticke, B., Haest, C.W.M. and Bergmann, W. (1982) Pflügers Arch. Eur. J. Physiol. 394, R28
17 Kosower, N.S. and Kosower, E.M. (1974) in Glutathione (Flohé, L., Benöhr, H.C., Sies, H., Waller, H.D. and Wendel, A., eds.), pp. 216–227, Georg Thieme Publishers, Stuttgart
18 Grunze, M., Forst, B. and Deuticke, B. (1980) Biochim. Biophys. Acta 600, 860–869
19 Deuticke, B., Rickert, I. and Beyer, E. (1978) Biochim. Biophys. Acta 507, 137–155
20 Bloch, R. (1973) Biochemistry 12, 4799–4801
21 La Celle, P. and Passow, H. (1971) J. Membrane Biol. 4, 270–283
22 Liu, T.-Y. (1977) in The Proteins, 3rd Edn. (H. Neurath, H. and Hill, R.L., eds.), Vol. 3, pp. 239–402, Academic Press, New York, San Francisco, London
23 Allen, D.W. and Cadman, S. (1976) Proc. Soc. Exp. Biol. Med. 152, 318–321
24 Haest, C.W.M., Kamp, D. and Deuticke, B. (1979) Biochim. Biophys. Acta 557, 363–371
25 Smith, R.P.P. and Ellman, G.L. (1973) J. Membrane Biol. 12, 177–188
26 Deuticke, B., Grunze, M., Forst, B. and Lütkemeier, P. (1981) J. Membrane Biol. 59, 45–55
27 Knauf, P.A. (1979) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 12, pp. 249–292, Academic Press, New York, London, Toronto, Sydney, San Francisco
28 Eaton, J.W. and Brewer, G.J. (1974) in The Red Blood Cell (Surgenor, D. MacN., ed.), Vol. 1, pp. 435–471, Academic Press, New York and London
29 Kosower, N.S., Zipser, Y. and Faltin, Z. (1982) Biochim. Biophys. Acta 691, 345–352
30 Bjerrum, P.J., Tranum-Hensen, J. and Mellgard, K. (1980) in Membrane Transport in Erythrocytes, Alfred Benzon Symposium 14 (Lassen, U.V., Ussing, H.H., Wieth, J.O., eds.), pp. 51–67, Munksgaard, Copenhagen
31 De Gier, J., Mandersloot, J.G. and Hupkes, J.V. (1971) Biochim. Biophys. Acta 233, 610–618
32 Jacob, H.S. and Jandl, J.H. (1961) J. Clin. Invest. 41, 779–792
33 Sutherland, R.M., Rothstein, A. and Weed, R.I. (1966) J. Cell. Physiol. 69, 185–198
34 Flohé, L., Niebch, G. and Reiber, H. (1971) Z. Klin. Chem. Klin. Biochem. 9, 431–437
35 Miller, A. and Smith, H.C. (1970) Br. J. Haematol. 19, 417–428
36 Harm, W. and Deamer, D.W. (1977) Physiol. Chem. Phys. 9, 501–512
37 Goldstein, B.D. and Harber, L.C. (1972) J. Clin. Invest. 51, 892–902
38 Deziel, M.R. and Girotti, A.W. (1980) J. Biol. Chem. 255, 8192–8198
39 Dubbelman, T.M.A.R., Haasnoot, C. and Van Steveninck, J. (1980) Biochim. Biophys. Acta 601, 220–227
40 Dubbelman, T.M.A.R., De Goeij, A.F.P.M. and Van Steveninck, J. (1980) Biochim. Biophys. Acta 595, 133–139
41 Kosower, N.S., Kosower, E.M. and Wertheim, B. (1969) Biochem. Biophys. Res. Commun. 37, 593–596
42 Kosower, N.S., Kosower, E.M. and Koppel, R.L. (1977) Eur. J. Biochem. 77, 529–534
43 Srivastava, S.K., Awasthi, Y.C. and Beutler, E. (1974) Biochem. J. 139, 289–295
44 Hosey, M.M., Plut, D.A. and Tao, M. (1978) Biochim. Biophys. Acta 506, 211–220
45 Scutari, G., Branca, D. and Pastorio, C. (1979) Boll. Soc. Ital. Biol. Sper. 55, 517–522
46 Haest, C.W.M. and Deuticke, B. (1975) Biochim. Biophys. Acta 401, 468–480
47 Pihl, A. and Lange, R. (1962) J. Biol. Chemistry 237, 1356–1362

48 Duhm, J., Deuticke, B. and Gerlach, E. (1969) Hoppe-Seyler's Z. Physiol. Chem. 350, 1008–1016
49 Haest, C.W.M. and Deuticke, B. (1976) Biochim. Biophys. Acta 436, 353–365
50 Gárdos, G. and Szasz, I. (1973) in Erythrocytes, Thrombocytes, Leucocytes. Recent Advances in Membrane and Metabolic Research (Gerlach, E., Moser, K., Deutsch, E., Wilmanns, W., eds.), pp. 31–33, Georg Thieme Publishers, Stuttgart
51 Branca, D., Scutari, G. and Siliprandi, N. (1978) J. Cell. Physiol. 95, 319–322
52 Kuratsin-Mills, J. and Lessin, L.S. (1981) Biochim. Biophys. Acta 641, 81–129
53 Schothorst, A.A., Van Steveninck, J., Went, L.N. and Suurmond, D. (1970) Clin. Chim. Acta 28, 41–49
54 Girotti, A.W. (1975) Biochemistry 14, 3377–3383
55 Dubbelman, T.M.A.R., De Goeij, A.F.P.M. and Van Steveninck, J. (1978) Photochem. Photobiol. 28, 197–304
56 De Goeij, A.F.P.M., Ververgaert, P.H.J.T. and Van Steveninck, J. (1975) Clin. Chim. Acta 62, 287–292
57 Deziel, M.R. and Girotti, A.W. (1982) Int. J. Biochem. 14, 263–266
58 Girotti, A.W. (1980) Biochim. Biophys. Acta 602, 45–56
59 Nakashima, K., Fujii, S. and Kaneko, T. (1980) Biomed. Res. 1, 548–551
60 Lorand, L., Siefring, G.E., Jr. and Lowe-Krentz, L. (1978) J. Supramol. Struct. 9, 427–440
61 Riemann, F., Zimmermann, U. and Pilwat, G. (1975) Biochim. Biophys. Acta 394, 449–462
62 Kinosita, K., Jr. and Tsong, T.Y. (1977) Nature 268, 438–441
63 Grinstein, S. and Rothstein, A. (1978) Biochem. Biophys. Acta 508, 236–245
64 Steck, Th.L. (1972) J. mol. Biol. 66, 295–305
65 Haglund, A.C. and Marsden, N.V.B. (1980) J. Polymer Sci. 18, 271–279
66 Ferry, J.D. (1937) J. Gen. Physiol. 20, 95–104
67 Lieber, R.M. and Steck, T.L. (1982) J. Biol. Chem. 257, 11651–11659
68 Lieber, R.M. and Steck, T.L. (1982) J. Biol. Chem. 257, 11660–11666
69 Stamatoff, J.B., Krimm, S. and Harvie, N.R. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 531–534
70 Dani, J.A. and Levitt, D.G. (1981) Biophys. J. 35, 501–508
71 Osberghaus, U., Schönert, H. and Deuticke, B. (1982) J. Membrane Biol. 68, 29–35
72 Nagle, J.F. and Scott, H.L., Jr. (1978) Biochim. Biophys. Acta 513, 236–243
73 Blok, E.M.C., Van der Neut-Kok, E.C.M., Van Deenen, L.L.M. and De Gier, J. (1975) Biochim. Biophys. Acta 406, 187–196
74 Taupin, C., Dvolaitzky, M. and Sauterey, C. (1975) Biochemistry 14, 4771–4775
75 Petrov, A.G., Mitov, M.D. and Derzbanski, A.I. (1980) in Advances in Liquid Crystal Research and Applications (Bata, L., ed.), pp. 695–737, Pergamon Press, Oxford and Akadémiai Kiadó, Budapest
76 Mandersloot, J.G., Gerritsen, W.J., Leunissen, B.J., Van Echteld, C.J.A., Noordam, P.C. and De Gier, J. (1981) Biochim. Biophys. Acta 640, 106–113
77 Noordam, P.C., Van Echteld, C.J.A., De Kruijff, B. and De Gier, J. (1981) Biochim. Biophys. Acta 646, 483–487
78 Israelachvili, J.N. (1977) Biochim. Biophys. Acta 469, 221–225
79 Tosteson, M.R., Lau, F. and Tosteson, D.C. (1973) Nature 243, 112–114
80 Gerritsen, W.J., Henricks, P.A.J., De Kruijff, B. and Van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 600, 607–619
81 Romans, A.Y., Allen, T.M., Meckes, W., Chiovetti, R., Jr., Sheng, L., Kercret, H. and Segrest, J.P. (1981) Biochim. Biophys. Acta 642, 135–148
82 Van der Steen, A.T.M., De Kruijff, B. and De Gier, J. (1982) Biochim. Biophys. Acta 691, 13–23
83 Van Zoelen, E.J.J., De Kruijff, B. and Van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 508, 97–108
84 Steck, T.L. (1978) J. Supramol. Struct. 8, 311–324
85 Tomita, M., Furthmayr, H. and Marchesi, V.T. (1978) Biochemistry 17, 4756–4770
86 Jordan, P.C. (1981) Biophys. Chem. 13, 203–212
87 Loewenstein, W.R. (1981) Physiol. Rev. 61, 829
88 Benz, R., Ishii, J. and Nakae, T. (1980) J. Membrane Biol. 56, 19–29
89 Freitag, H., Neupert, W. and Benz, R. (1982) Eur. J. Biochem. 123, 629–639
90 Savitz, D. and Solomon, A.K. (1971) J. Gen. Physiol. 58, 259–266
91 Sha'afi, R.I. (1977) in Membrane Transport in Red Cells (Ellory, J.C. and Lew, V.L., eds.), pp. 39–52, Academic Press, London, New York, San Francisco
92 Schultz, S.G. and Solomon, A.K. (1961) J. Gen. Physiol. 44, 1189–1199
93 Monk, C.B. (1961) Electrolytic Dissociation, p. 271, Academic Press, London and New York
94 Sober, H.A. (ed.) (1970) Handbook of Biochemistry, 2nd Edn., The Chemical Rubber Co., Cleveland
95 Stein, W.D. (1967) The Movement of Molecules across Cell Membranes, Academic Press, New York and London