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LABELING OF HUMAN ERYTHROCYTE MEMBRANES WITH EOSIN PROBES USED FOR PROTEIN DIFFUSION MEASUREMENTS

INHIBITION OF ANION TRANSPORT AND PHOTO-OXIDATIVE INACTIVATION OF ACETYLCHOLINESTERASE

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Summary

The binding of eosin-isothiocyanate (eosin-NCS) and iodoacetamido-eosin (IA-eosin) to band 3 proteins in the membrane of human erythrocytes is characterized by studying the effect of these probes on the anion transport system. Although the unbrominated fluorescein precursors do not affect anion transport, both eosin labels are strong inhibitors of sulphate exchange in intact erythrocytes. 50% inhibition is obtained by binding $4.7 \cdot 10^5$ or $6.0 \cdot 10^5$ molecules/cell for eosin-NCS and IA-eosin, respectively. Both eosin probes are irreversibly bound and occupy common binding sites with 4,4′-diisothiocyano-1,2-diphenyl-ethane-2,2′-disulfonic acid (H_2DIDS), although other sites are labeled as well. The inhibition of anion transport is light independent and can therefore not be attributed to a photosensitizing action of the eosin probes. Both eosin derivatives, however, inactivate acetylcholinesterase upon illumination of air-equilibrated samples of hemoglobin-free labeled ghosts. The inactivation of the enzyme is accompanied by the formation of protein aggregates as visualized by polyacrylamide gel electrophoresis. These effects are not observed when intact erythrocytes are illuminated in the presence of eosin probes suggesting a protective effect of hemoglobin during the labeling procedure. Protection of ghosts from photo-oxidation is achieved by displacing air with argon. These results are discussed in relation to the use of these and similar probes to measure protein diffusion in membranes.

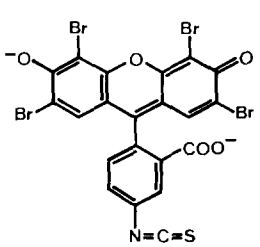
Abbreviations: eosin-NCS, eosin-5-isothiocyanate; IA-eosin; 5-iodoacetamido-eosin; fluorescein-NCS, fluorescein-5-isothiocyanate; DIDS, 4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid; H_2DIDS, 4,4′-diisothiocyano-1,2-diphenyl-ethane-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyano-2,2′-stilbene-disulfonic acid; SDS, sodium dodecyl sulphate.

Introduction

A technique for measuring the rotational diffusion of membrane proteins has been developed in recent years [1—4]. The method exploits the long lifetime of triplet states to measure the relatively slow rotation which is anticipated for integral membrane proteins. Rotational diffusion is measured by observing the decay of dichroism of flash-induced absorption transients of a probe molecule. A detailed description of this spectroscopic method is given elsewhere [5]. The triplet probe eosin has been shown to be suitable for measuring rotational diffusion using the flash-photolysis method [1—3]. Two reactive derivatives, eosin-5-isothiocyanate (eosin-NCS) (Fig. 1, I) and 5-iodoacetamido-eosin (IA-eosin) (Fig. 1, II), enable the dye to be covalently attached to membrane components [2,3,5], interacting preferentially with amino- or sulphydryl-residues, respectively.

When human erythrocytes are labeled with eosin-NCS, it has been shown [6,27] that most of the label is attached to a major membrane-spanning glycoprotein known as band 3 (nomenclature according to Fairbanks et al. [7]). The application of eosin-NCS to measure rotational diffusion of band 3 proteins has been reported [6]. More recently, we have found that similar results are obtained with the IA-eosin derivative [8].

In the present study the interaction of the two eosin probes with the erythrocyte membrane is further characterized emphasizing two properties of the probes which may be derived from their molecular structures: (i) At physiological pH the eosin derivatives are di-anions and might therefore be expected to interact with the anion transport system of the erythrocyte. There is now strong evidence that band 3 proteins are involved in anion transport across the erythrocyte membrane [9—15]. Here we have studied the influence of the eosin probes on sulphate-exchange. (ii) The high yield of the eosin triplet-state makes these probes not only useful for flash photolysis measurements but at the same time results in their acting as powerful photo-sensitizing agents [16,17]. This raises the question of whether photochemical damage to the membrane occurs during the flash experiments. Among the enzymatic functions associated with the red cell membrane [18] we chose acetylcholinesterase (EC 3.1.1.7) activity as a marker for investigating photodynamic actions of eosin derivatives.



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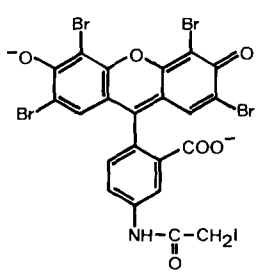

II

Fig. 1. Triplet probes: (I) eosin-5-isothiocyanate; (II) 5-iodoacetamido-eosin.

Materials and Methods

Materials

Eosin-NCS and IA-eosin were either prepared as described previously [2,5] or purchased from Molecular Probes Inc., Roseville, MN. Iodoacetamido-fluorescein was from Molecular Probes, fluorescein-isothiocyanate (fluorescein-NCS) from Serva. Eosin (disodium salt) was obtained from Koch Light Lab., and fluorescein (disodium salt) from Merck. 4,4′-Diisothiocyano-1,2-diphenyl-ethane-2,2′-disulfonic acid (H_2DIDS) was a kind gift of Professor H. Passow. $Na_2^{35}SO_4$ was from Amersham Radiochemical Centre Ltd., U.K. Bovine serum albumin (fraction V) was from Fluka AG, Switzerland, and showed good binding capacities for eosin. Acrylamide and bisacrylamide were from Serva. Other chemicals were analytical grade.

Labeling of erythrocytes and ghost preparation

Fresh human blood (O^+) was obtained from the Swiss Red Cross Blood Transfusion Service and used within 24 h. The blood was centrifuged and the plasma and white cells removed by aspiration. The erythrocytes were then washed 2—3 times in 5 mM NaH_2PO_4/Na_2HPO_4, 150 mM NaCl, pH 7.4.

Eosin derivatives were first dissolved in isotonic phosphate buffer (pH 7.4) at a concentration of 0.5 mg/ml. 1 mg of label was added/5 ml of packed erythrocytes and the reaction allowed to proceed for 3 h at room temperature in the dark. Unreacted label was removed by two further washes of the cells in a 40—50 fold volume of isotonic buffer. The cells were then hemolysed in 40—50 volumes of 5 mM NaH_2PO_4/Na_2HPO_4, pH 7.4. The ghosts were sedimented and washed 3—4 times with the same buffer. All operations except the labeling step were carried out at 0—4° C.

Reduction of eosin binding by prior H_2DIDS labeling

A 4-fold dilute suspension of packed erythrocytes was incubated in the presence or absence of H_2DIDS (50 μM) for 30 min at 37° C in the dark. Cells were then washed 2 times in 50 mM Tris, 50 mM Na_2SO_4, 55 mM NaCl, pH 7.4. After a further wash in the same buffer containing 0.5% bovine serum albumin the cells were finally washed 2 times in 20 mM NaH_2PO_4/Na_2HPO_4, 50 mM Na_2SO_4, 55 mM NaCl, pH 7.4 and hematocrit of the labeled and unlabeled cells was adjusted to the same value between 60 and 70%. Aliquots of the cells were then exposed to eosin derivatives as described above.

Sulphate exchange in intact erythrocytes

Erythrocytes were washed in 20 mM NaH_2PO_4/Na_2HPO_4, 50 mM Na_2SO_4, 55 mM NaCl (pH 7.4) and equilibrated with $Na_2^{35}SO_4$ for 2 h at 37° C. They were then exposed to eosin labels as described above. After simultaneous removal of external tracer and unreacted label by washes at 0—4° C in the same medium, the cells were resuspended in a 15-fold excess of pre-warmed medium. At different times aliquots (generally in duplicate) were withdrawn from the cell suspension and centrifuged for 1 min in a bench centrifuge. Although hemolysis was negligible the supernatants were routinely deproteinized with an equal volume of 8% trichloroacetic acid and radioactivity was determined by

liquid scintillation counting. The supernatant counts at the time of isotopic equilibration were estimated from aliquots of deproteinized cell suspensions. All transport experiments were carried out at 37° C unless specified otherwise.

Analysis of transport kinetics

The time course of release of $^{35}SO_4$ from $^{35}SO_4$ preloaded erythrocytes at Donnan equilibrium is a first-order process:

$$\ln\{[\text{cpm}(\infty) - \text{cpm}(t)]/\text{cpm}(\infty)\} = -k_i t \quad (1)$$

where cpm(∞), cpm(t) represent the total counts/min of $^{35}SO_4$ in the medium at the time of isotopic equilibration and at time t, respectively, k_i is the rate constant. Plotting of $[\text{cpm}(\infty) - \text{cpm}(t)]/\text{cpm}(\infty)$ versus t on a semilogarithmic scale thus results in a straight line with slope $-k_i$.

Percent inhibition is obtained from the following equation:

$$\% \text{ inhibition} = (1 - k_i/k_{\text{control}}) \times 100 \quad (2)$$

Light-protection experiments

To exclude any photochemical reaction mediated by eosin probes during preparations under normal laboratory light conditions, special precautions were taken in some experiments to protect the samples against light. Such experiments were carried out under a dim red illumination (no light emission below 600 nm) whenever a weak illumination was necessary to ensure safe handling of the samples. Otherwise they were kept in complete darkness.

Illumination-experiments

Illuminations were carried out at 4° C with a Leitz slide-projector (500 W lamp). Glass tubes (diameter 14 mm) or glass-cuvettes (diameter 10 mm) containing the samples were placed at a distance of 25 cm from the front lens of the projector (light intensity 5 ± 1 mW/cm^2). The samples were either open to air and well stirred to allow oxygen saturation of the solution or they were sealed after bubbling the solutions for 5—20 min with argon passed through an Oxysorb (Messer Griesheim, Düsseldorf).

Analytical procedures

The amount of bound eosin was determined spectrophotometrically as described previously after first solubilising ghosts with sodium dodecyl sulphate (SDS) [2]. Protein was determined using the method of Lowry et al. [19]. Acetylcholinesterase activity was measured by the method of Ellmann et al. [20] following a procedure of Steck and Kant [21].

SDS-polyacrylamide gel electrophoresis

Ghosts were first solubilised for 5 min at 95° C in 1 volume of 2% SDS. Before electrophoresis the samples were reduced by incubation for 40 min at 37° C in 0.2 volumes of 0.29 M Tris-HCl, pH 6.8, 25% (v/v) glycerol, 10% (w/v) SDS, 200 mM dithioerythritol and bromphenolblue as tracking dye. The electrophoresis was run on a discontinuous slab gel (thickness 2 mm) having the following characteristics [22]: Running gel: 8% acrylamide, 0.38 M Tris-HCl,

0.1% SDS, pH 8.8. Sampling gel: 3.6% acrylamide, 0.067 M Tris-HCl, 0.1% SDS, pH 6.8. The bisacrylamide was maintained at 2.7% of the acrylamide monomer concentration. The electrophoresis buffer consisted of 0.027 M Tris, 0.2 M glycine, 0.1% SDS. The current applied was 10—13 mA/cm^2. The gels were stained with Coomassie brilliant blue.

Results and Discussion

Inhibition of sulphate exchange in intact erythrocytes

Efficiency of eosin derivatives. Both eosin-NCS and IA-eosin strongly inhibit sulphate exchange in labeled erythrocytes as measured by tracer efflux under equilibrium conditions (Fig. 2). A calculation of the number of molecules required to produce 50% inhibition shows that in this range the monofunctional eosin probes are of comparable efficiency to the frequently used difunctional disulfonic acid stilbene inhibitors DIDS and H_2DIDS [23]. Using the conversion factor of $6.0 \cdot 10^{-10}$ mg protein/ghost [24] eosin derivatives inhibit sulphate exchange to 50% at $4.7 \cdot 10^5$ and $6.0 \cdot 10^5$ molecules/cell for eosin-NCS and IA-eosin, respectively.

A difference between the eosin probes and DIDS is that increased labeling with the latter eventually produces close to 100% inhibition of transport. With eosin probes, labeling erythrocytes at room temperature and pH 7.4 for up to 3 h failed to produce more than about 80% inhibition. A possible explanation is that the probes become less specific for band 3 at higher labeling strengths. However, other probes such as 2-(4′-amino phenyl)-6-methylbenzene-thiazol-3,7′-disulfonic acid are also found to produce incomplete inhibition [25], although the explanation is unclear.

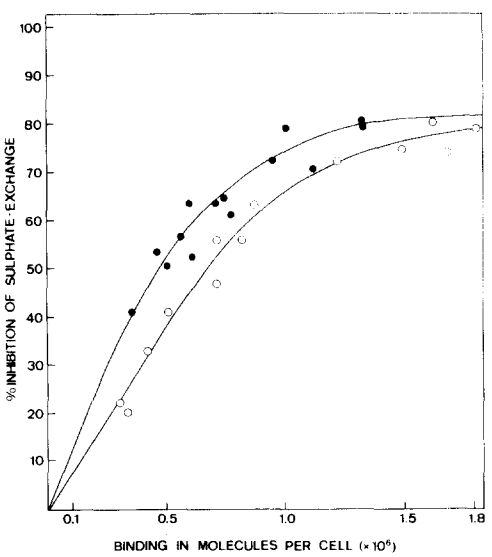

Fig. 2. Inhibition of sulphate exchange by covalent eosin labeling of erythrocytes: Sulphate exchange in intact erythrocytes was measured as described in Materials and Methods. Bound eosin label was determined in ghosts prepared from aliquots of the cells. Results are collected from 7 experiments performed on different blood samples. ●, eosin-NCS; ○, IA-eosin.

TABLE I

REDUCTION OF EOSIN BINDING BY PRIOR H_2DIDS LABELING

Data indicate the labeling strength (molecules/cell [$\times 10^{-6}$]) from two separate experiments.

	Eosin-NCS		IA-eosin	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Without prior H_2DIDS labeling	1.33	1.01	1.68	1.40
After prior H_2DIDS labeling	0.54	0.53	0.84	0.87
Percent reduction	59	48	50	38

As can be seen from Fig. 2, eosin-NCS is slightly more efficient than IA-eosin. Consistent with the current opinion that NH_2- rather than SH-groups are involved in anion transport [9,26], it could be argued that a higher reactivity of the iodoacetamido compound towards sulphydryl residues leads to binding of the label on sites unrelated to anion transport but still on band 3. It is however at the present time difficult to ascertain that both eosin probes show the same selectivity towards band 3 proteins since label distributions are determined by methods with limited resolution (fluorimetrically in gel slices or spectrophotometrically in selective extractions [27]). Therefore it cannot be ruled out that a lower specificity of IA-eosin towards band 3 might contribute to the lower inhibition induced by this probe.

When cells were pretreated with H_2DIDS under conditions which yielded more than 95% inhibition of the anion transport system, the amount of eosin derivatives which could be further bound was roughly 2 fold reduced (Table I). This suggests that there are common binding sites with H_2DIDS for both eosin labels but there are other sites available as well.

Irreversibility of eosin-induced inhibition. For some of the disulfonic acid stilbene inhibitors (i.e., 4-acetamido-4′-isothiocyano-2,2′-stilbene-disulfonic acid (SITS)) it was shown that part of the inhibition observed in labeled and extensively washed erythrocytes could be readily reversed by washing the cells in buffer containing serum albumin [9]. In the case of eosin-NCS and IA-eosin, however, the inhibition cannot be reversed by repeated washing of labeled erythrocytes in buffer containing 0.5% serum albumin (Fig. 3) hence indicating an irreversible nature of the binding.

Further evidence that the binding is irreversible comes from the fact that inhibition is increased with prolonged exposure of the cells to a constant amount of the inhibitors even in the absence of albumin in the washing buffer (Fig. 4). Since reversible inhibition caused by the disodium salt of eosin is complete within a few minutes after addition of the inhibitor to the cells (see below), we would expect the reversible binding of the covalent probes also to be complete within a short time and therefore the observed inhibition should be independent of further prolongation of the labeling time. In the case of SITS it was shown in fact by Cabantchik and Rothstein [9] that the inhibition was independent of labeling time as long as the reversible component of the binding was not abolished by washing the cells in buffer containing albumin.

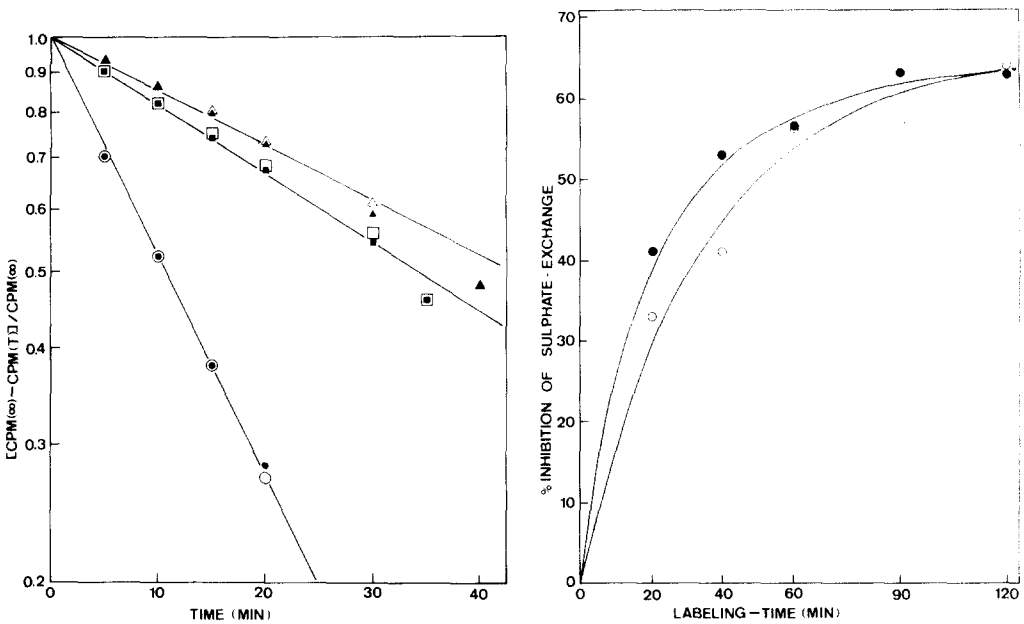

Fig. 3. Independence of eosin-induced inhibition from washing with buffered albumin: After tracer equilibration and eosin labeling, aliquots of the cells were washed once in 20 mM NaH_2PO_4/Na_2HPO_4, 50 mM Na_2SO_4, 55 mM NaCl pH 7.4, then 2 times in the same buffer with or without 0.5% bovine serum albumin and a fourth time in the same buffer free of albumin. Sulphate exchange was measured as described in Materials and Methods. Open symbols, buffer free of albumin; closed symbols, buffer containing albumin; ○/●, unlabeled cells; □/■, eosin-NCS; △/▲, IA-eosin. Labeling strengths: $1.1 \cdot 10^6$ molecules eosin-NCS/cell; $1.5 \cdot 10^6$ molecules IA-eosin/cell.

Fig. 4. Dependence of sulphate-exchange inhibition on labeling time: Washed erythrocytes were equilibrated with $^{35}SO_4$ and exposed for various times to a constant concentration (0.2 mM) of the eosin labels. At various times aliquots of the labeling suspensions were withdrawn and cells were immediately washed in a 50-fold volume of ice-cold buffer to stop the labeling reaction. The cells were diluted and kept at 0—4°C until immediately prior to sulphate-exchange measurements. All samples were washed a further time in a common centrifugation step. Unlabeled control samples were taken together with the 20-min and the 120-min samples. All washing buffers were free of albumin. Labeling strengths were determined in ghosts and varied from $3.6 \cdot 10^5$ to $6.6 \cdot 10^5$ molecules bound/cell for eosin-NCS (●) and from $4.3 \cdot 10^5$ to $8.7 \cdot 10^5$ molecules bound/cell for IA-eosin (○).

Comparison of the eosin probes with fluorescein derivatives. Cabantchik and Rothstein [9] have reported that fluorescein-NCS did not inhibit anion transport. Because of the similarity in structure between fluorescein and eosin, we measured sulphate exchange in erythrocytes labeled with fluorescein derivatives under the same labeling conditions normally used for eosin probes. These conditions are considerably more drastic than those used by Cabantchik and Rothstein where labeling was for 30 min at 5°C. By measuring the absorbance at 495 nm of fluorescein-labeled ghosts we were able to make an estimate of the amount of fluorescein bound. At labeling strengths up to $3.0 \cdot 10^5$ molecules/ghost no inhibition could be detected.

We then further compared the influence of the disodium salts of eosin and fluorescein when present in the medium during the tracer efflux. Fig. 5 shows that eosin strongly inhibits sulphate exchange, demonstrating that inhibition

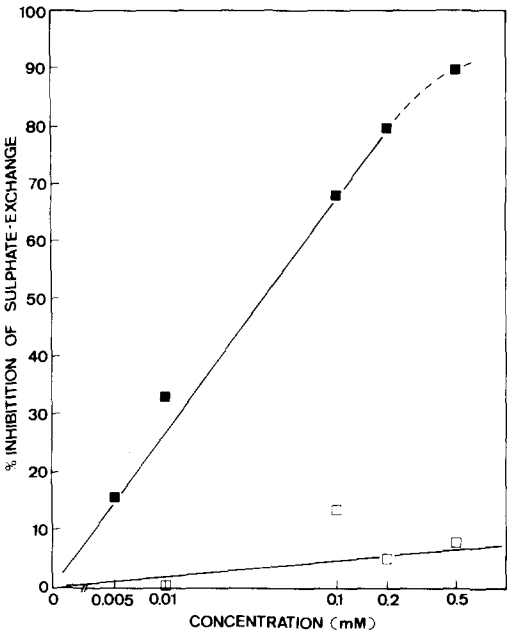

Fig. 5. Comparison of the effects on sulphate permeability of eosin and fluorescein: $^{35}SO_4$-loaded and washed erythrocytes were resuspended in pre-warmed medium containing different concentrations of eosin (■) or fluorescein (□) (disodium salts) at pH 7.4. Fluxes were immediately measured as described in Materials and Methods.

can also be produced by non-covalent binding. Fluorescein, on the other hand, was without significant effect. This result is interesting since the molecular structures of fluorescein and eosin differ only in the presence of 4 bromine atoms in the latter. It is conceivable that differences in size or hydrophobicity between eosin and fluorescein might influence their binding or orientation at the anion transport system. However it is also possible that a difference in anionic character might account for the different behaviour of the two probes towards the anion transport system. It has been suggested [9] that the failure of fluorescein-NCS to produce inhibition of anion transport might be attributed to a lacking anionic characteristic of this amino reagent. Due to the presence of bromine atoms in ortho positions to phenolic hydroxyl groups, the pK_a value (4.3) for the monoanion of eosin [28] actually is considerably lower than the corresponding pK_a value (6.7) for fluorescein [28,29]. The effective charge of the dyes could be further affected by a difference between the local pH of the anion transport system and the bulk pH. An investigation of the pH dependence of the action of different halogenated fluorescein compounds on anion transport might therefore provide some information about the molecular architecture of the anion transport system in human erythrocytes.

Photodynamic action of eosin derivatives

Non photo-oxidative inhibition of the anion transport system. The efficiency of fluorescein derivatives as photosensitizing agents is known to increase with

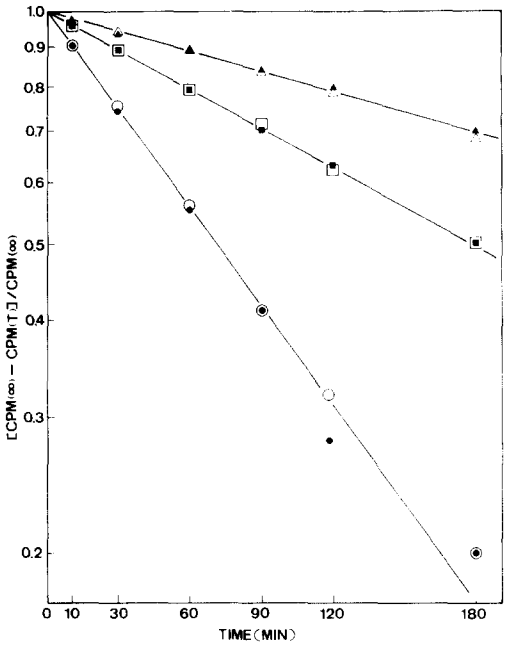

Fig. 6. Light independence of eosin-induced sulphate-exchange inhibition: After equilibration of erythrocytes with $^{35}SO_4$ all steps except the illumination itself were carried out under red light. The cells were labeled with eosin derivatives for 3 h at room temperature in the dark. They were then left for 20 min at 4° C before aliquots of the labeling suspensions were withdrawn and illuminated for 1 h at 4° C as described in Materials and Methods. The rest of the suspensions were kept in the dark during this time. The cells were then washed and sulphate exchange was measured at 25° C. Open symbols, light protected samples; closed symbols, illuminated samples; ○/●, unlabeled cells; □/■, eosin-NCS; △/▲, IA-eosin. Labeling strengths: $0.8 \cdot 10^6$ molecules eosin-NCS/cell; $1.6 \cdot 10^6$ molecules IA-eosin/cell.

the introduction of halogen atoms into the fluorescein molecule [16]. Since eosin probes are strong inhibitors of the anion transport system in human erythrocytes whereas their fluorescein analogues are without any significant effect, the question arises whether the inhibition induced by eosin might be due to photo-oxidative damage of the anion transport system. If this would be the case, one would expect that the eosin-induced inhibition might be enhanced by illumination of the erythrocytes during the labeling procedure. However, Fig. 6 shows that at relatively high labeling strengths the inhibition observed in illuminated samples does not differ to any measurable extent from their corresponding control samples which were protected from light throughout the experiment. It was further observed that even at very low labeling strengths, the measurable inhibition (10—30%) was not abolished by protection of the samples from light.

A further control consisted in the following experiment: erythrocytes were incubated with the disodium salt of eosin under the same conditions as for normal labeling. After illumination of these samples the eosin was removed by washing the cells in buffer containing serum albumin. Sulphate exchange studies on these samples showed no detectable inhibition of sulphate transport.

Photo-oxidative inactivation of acetylcholinesterase. Illumination of intact

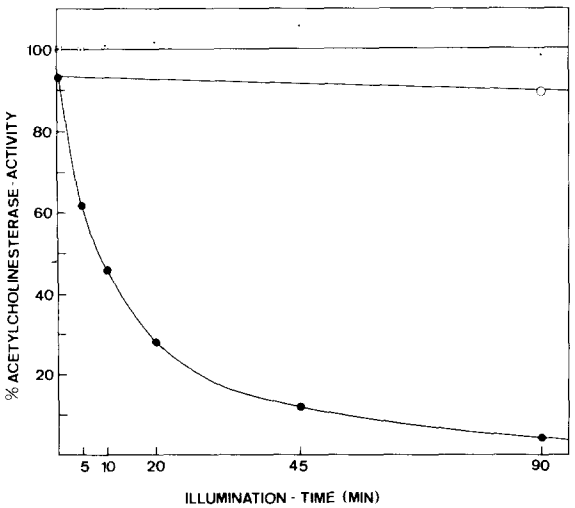

Fig. 7. Photo-oxidative inactivation of acetylcholinesterase in eosin-NCS-labeled ghosts: Except for the illumination step the entire experiment starting with washed erythrocytes was carried out under red light. Ghosts were prepared and diluted 4 times with 5 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) to a final concentration of 1.2 mg membrane protein/ml. At various times after starting illumination, aliquots were withdrawn from the cuvettes for determination of acetylcholinesterase activity. The glass cuvettes (width 10 mm) were either open to air and well stirred or sealed after displacement of oxygen by argon. Qualitatively the same results were obtained using IA-eosin instead of eosin-NCS. □, unlabeled ghosts (air); ●, eosin-NCS (air); ○, eosin-NCS (argon). Labeling strength: $9.5 \cdot 10^5$ molecules/cell.

erythrocytes during the labeling procedure did not produce any significant inactivation of the membrane-associated enzyme acetylcholinesterase. On illumination of eosin-labeled hemoglobin-free ghosts, however, both eosin labels inactivate acetylcholinesterase in air-equilibrated samples (Fig. 7). SDS-polyacrylamide gel electrophoresis shows that the inactivation of acetylcholinesterase is accompanied by extensive formation of protein aggregates which do not enter SDS gels (Fig. 8). However, it seems that the most prominent contribution to these aggregates derives from spectrin (bands 1 and 2 [7]) whereas band 3 as well as some other bands are less affected.

The difference between intact erythrocytes and ghosts could be due either to a protective action of hemoglobin or to the presence of protective agents in the intact cells. Catalase and superoxide dismutase as well as glutathione peroxidase counteract reactive oxygen species, namely peroxides and superoxide radicals. However it is likely that singlet oxygen or even hydroxyl radicals are generated during eosin-mediated photo-oxidation. So far no enzyme has been described which could protect the erythrocyte against these highly reactive species. It should further be noted that cross-linking of membrane proteins is not due to disulfide-bridge formation since aggregation on polyacrylamide gels is observed after reduction of the samples with dithioerythritol prior to electrophoresis. Thus, the protection of SH-groups by glutathione in the intact erythrocyte cannot be responsible for the observed difference between intact cells and ghosts. It therefore appears probable that the different susceptibility of intact cells and ghosts should primarily be attributed to a protective action of hemoglobin. Since hemoglobin has an

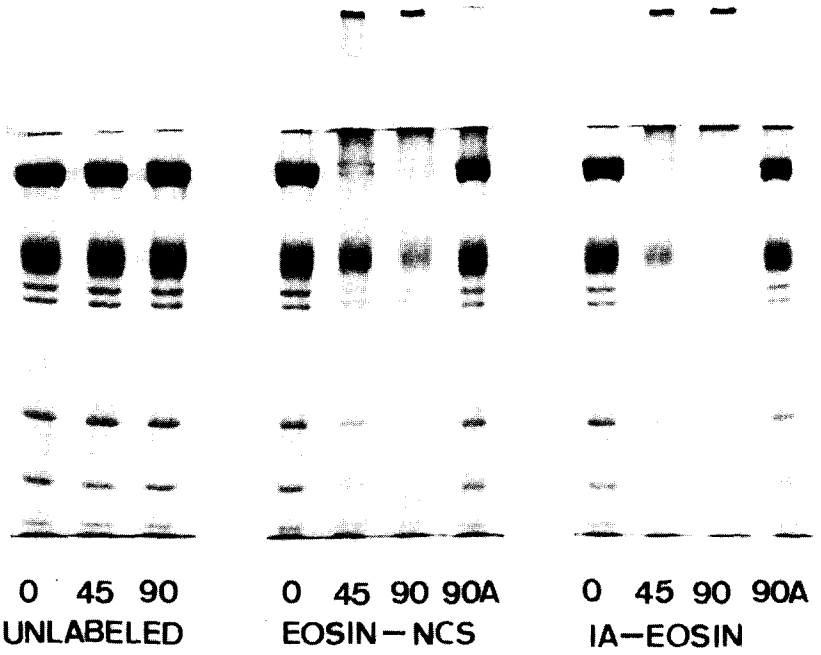

Fig. 8. Light-dependent aggregation of membrane proteins in eosin-labeled ghosts: Labeled or unlabeled ghosts were illuminated for the indicated time periods (min), either in the presence of air or after displacement of air by argon (indicated by the letter A). Ghosts were solubilised and applied to electrophoresis as described in Materials and Methods, 15 μg protein/gel. Labeling strengths: $0.95 \cdot 10^6$ molecules eosin-NCS/cell; $1.25 \cdot 10^6$ molecules IA-eosin/cell.

absorption band overlying the absorption maxima of the eosin probes, it will act as a blocking filter for those wavelengths which cause eosin-mediated photo-oxidation.

In agreement with previous reports [16,30] we found that the photo-oxidative process is not directly dependent on covalent binding of the sensitizer to the membrane but depends mainly on the efficiency of triplet state formation. Eosin without a reactive group produces the same aggregation of proteins whereas fluorescein with a much lower yield in triplet state formation [31,32] does not (Fig. 9).

The observation of photo-oxidative damage in ghosts indicates that some care must be taken in handling eosin-labeled hemoglobin-free erythrocyte ghosts. Although under normal laboratory light conditions the photochemical reactions mediated by eosin are slow, prolonged exposure of the samples to strong light should be avoided.

Under an argon atmosphere, the enzyme activity is almost completely retained, even after long illumination times (Fig. 7). The formation of protein aggregates is also prevented (Figs. 8 and 9). Measurements of rotational diffusion by flash photolysis are routinely performed with ghost suspensions in which air is displaced by argon [5,6]. Tests on samples used for flash photolysis experiments show that as judged by SDS-polyacrylamide gel electro-

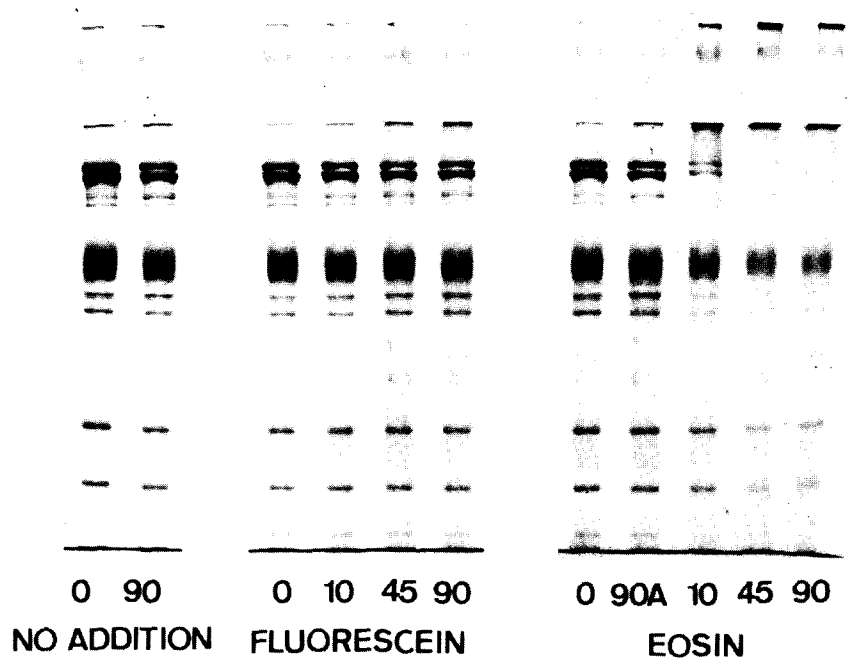

Fig. 9. Efficiency of eosin and fluorescein to mediate light-dependent aggregation of ghost-membrane proteins: Unlabeled ghosts were illuminated in the presence or absence of eosin or fluorescein (disodium salts, $3.0 \cdot 10^{-6}$ M) for the indicated time periods (min). The samples were either open and well stirred to allow air saturation or well sealed after displacement of air by argon (indicated by the letter A). Ghosts were solubilised and applied to electrophoresis as described in Materials and Methods, 12 μg proteing/gel.

phoresis and acetylcholinesterase activity, no damage to the membrane occurs under the conditions of rotational diffusion measurements.

Finally, a different method, that of fluorescence photobleaching recovery, has been used to detect the lateral diffusion of membrane components [33—37]. In this method, intense illumination is used to bleach chromophores such as fluorescein and rhodamine. It should be noted that the intensity of illumination in the experiments described here produced only limited bleaching of eosin, even after 90 min illumination. Although fluorescein was found to be much less effective in mediating photo-oxidation than eosin, this is not necessarily advantageous, since the rate of bleaching is also reduced. In both cases, this is due to the lower triplet yield of fluorescein compared with eosin. Thus the possibility that bleaching may, at least in some instances, be accompanied by membrane damage should perhaps be further considered in the light of the experiments with erythrocyte membranes reported here.

Noted added in proof (received November 6th, 1978)

After submission of this paper, we found that a third eosin derivative, eosin-5-maleimide, produces 100% inhibition of sulphate exchange after binding $1.1 \cdot 10^6$ molecules/cell. This number is in excellent agreement with the latest values published for the most powerful disulfonic acid stilbene inhibitors.

Acknowledgements

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