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PHOTO-ACTIVATED INHIBITION OF SULFATE EQUILIBRIUM EXCHANGE IN HUMAN ERYTHROCYTE GHOSTS BY A 4-AZIDO-2-NITROBENZOATE DERIVATIVE OF PHLORIZIN

JACK H. KAPLAN* and HUGO FASOLD

Max-Planck-Institut für Biophysik and Institut für Biochemie der Universität, Frankfurt/M (G.F.R.)

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SUMMARY

Like phlorizin, two glycosidic esters of phlorizin, the 4-azido-2-nitrobenzoate (ANB-phlorizin) and the 2-nitrobenzoate (NB-phlorizin) were found to be effective inhibitors of SO_4^{2-} equilibrium exchange at the outer but not at the inner membrane surface of the human erythrocyte ghost. After photolysis of ghost suspensions in the presence of extracellular ANB-phlorizin an irreversible inhibition of SO_4^{2-} exchange was observed, while photolysis of intracellular ANB-phlorizin was without effect. After photolysis in the presence of extracellular or intracellular tritiated ANB-phlorizin gel electrophoresis of the labelled membranes revealed similar locations of binding. These findings suggest that the sidedness of action of ANB-phlorizin could not be related to inaccessibility of the inner membrane surface for the agent but that inhibition occurs via binding to fixed sites at the outer membrane surface that are not associated with a mobile carrier which crosses the membrane.

INTRODUCTION

The study of the sidedness of the action of an inhibitor of transport across a membrane is a prerequisite for an understanding of its mode of action. If inhibition can be brought about at either surface of the membrane, the effect could be on a site of a mobile carrier or on fixed sites located in both membrane surfaces. If inhibition is confined to one surface only, it is likely that a reaction with an immobile site is involved. Previous work with phlorizin has shown that this agent inhibits anion exchange at the outer but not at the inner surface of the red blood cell ghosts [1, 2]. Although the successful incorporation of the inhibitor into ghosts was demonstrated by direct determination of the trapped agent and by its inhibition of sugar transport, the question remained open whether or not the inner membrane surface is as easily

Abbreviations: ANB-phlorizin, 4-azido-2-nitrobenzoate glycoside of phlorizin; NB-phlorizin, 2-nitrobenzoate glycoside of phlorizin; DAS, 4,4'-diacetamidostilbene-2,2'-disulfonic acid.

* Present address: Dept. of Physiology, Yale University, Medical School, New Haven, Conn. 06510, U.S.A.

accessible to the agent as the outer membrane surface. In order to investigate the accessibility of the cytoplasmic membrane surface we incorporated into red cell ghosts a tritiated photo-reactive derivative of phlorizin. After resealing in the dark, the reversibly bound agent could be removed from the extracellular medium by washing. Subsequently the ghosts were exposed to light and the effects on SO_4^{2-} equilibrium exchange and the amount of irreversible fixation of the photoreactive agent were measured.

MATERIALS AND METHODS

The compound utilized in the present studies is a 4-azido-2-nitrobenzoate derivative of phlorizin (ANB-phlorizin). Synthesis of the radioactively labelled reagent was achieved as follows.

Phlorizin (10.5 mg) was dissolved in ethanol (0.8 ml) containing 2 mCi of [3H]phlorizin (NEN Chemicals). The solution was evaporated to dryness under reduced pressure (approx. 10 Torr). Last traces of ethanol were removed by repeated washing and reevaporation using dry pyridine. The residue was then dissolved in dry pyridine (1.5 ml) and 2-nitro-4-azidobenzoyl chloride (8 mg) was added. The vessel was immediately tightly closed and the mixture stirred until the acid chloride crystals had dissolved. The solution was then left to stand over-night and thereafter evaporated to dryness under reduced pressure. The residue was suspended in about 2 ml of water and sufficient sodium bicarbonate added to ensure a slightly alkaline pH. The suspension was then extracted with ethyl acetate (3×0.5 ml). The combined ethyl acetate extracts were evaporated under reduced pressure and the pale yellow residue redissolved in ethyl acetate (0.3 ml). This solution was then applied to a silica gel (granular form, Woelm Co.) column (0.5×20 cm), pre-equilibrated with methylene chloride/methanol (95: 5, v/v). The column was eluted with methylene chloride/methanol (9: 1, v/v). Fractions of 1–4 ml were taken using an automatic fraction collector (LKB). The fractions were then monitored by thin-layer chromatography and scanned with the aid of a BF thin-layer counter. The first fractions usually contained small amounts of phlorizin bearing more than one nitrobenzoate residue. The main peak comprised the monobenzoylated derivative and finally a low broad peak of phlorizin was eluted. The monobenzoate derivative was characterized from its NMR spectrum and elemental analysis. Phlorizin could be liberated from the monobenzoylated derivative after a short incubation in 2 M caustic soda. The de-azo analog (hereafter NB-phlorizin) used in these studies was synthesized following the same procedure using 2-nitrobenzoyl chloride. Quantitative assays of phlorizin and its purified derivatives were made using the Lowry procedure [3] for proteins. Calibration curves were established between 0.01 and 0.1 mg of the substances and in these ranges were linear. The precise location of the 4-azido-2-nitrobenzoate moiety on the glucose molecule of phlorizin is not yet unambiguously established.

Experiments involving the photo-activated inhibition were carried out as follows: the resealed erythrocyte ghosts were prepared as described previously [4], containing 130 mM NaCl, 10 mM Na_2SO_4, 20 mM Tris · Cl pH 7.2, a trace of $^{35}SO_4^{2-}$, and, if desired, the photo-reactive agent. The hemoglobin concentration of the ghosts was about 5 % of that in the cells from which they were derived. The resealed ghosts were illuminated in the presence or absence of ANB-phlorizin in a

medium of the composition indicated above. Illumination was performed in quartz tubes contained in a water-cooled holder using a Hanau 500 W ultraviolet light source with a Corning WG 320 filter between the light source and quartz tubes. After photolysis the cells were washed 7 times in ice-cold medium to remove extracellular $^{35}SO_4^{2-}$ and those products of photolysis which did not irreversibly combine with the membrane. Subsequently, the rate of efflux of $^{35}SO_4^{2-}$ into the medium described above was measured at 30 °C by following the appearance of $^{35}SO_4$ in the supernatant. Rate constants for $^{35}SO_4$ efflux were obtained by dividing the initial slope of the curves relating the appearance of $^{35}SO_4^{2-}$ in the extracellular medium by the amount of $^{35}SO_4^{2-}$ present in the medium at infinite time. Samples containing $^{35}SO_4^{2-}$ were mixed in scintillation fluid (Instagel, Packard) and counted in a Packard Tricarb Liquid Scintillation Spectrometer. Control experiments showed that the rate of $^{35}SO_4^{2-}$ exchange was unaffected by exposure of $^{35}SO_4^{2-}$ loaded ghosts to light in the absence of ANB-phlorizin.

In experiments where the radioactively labelled ANB-phlorizin was used, after photolysis and washing, hemoglobin-free ghosts were prepared from an aliquot of the same ghosts used in the determination of the rate of $^{35}SO_4^{2-}$ efflux. The ghosts were rehemolyzed in 20-times their own volume of a solution containing 0.1 % saponin/10 mM KCl/5 mM EDTA, pH 7.0 at 0° C, and washed in this medium until free of hemoglobin. The white ghosts were then dissolved in 5 % sodium dodecyl sulphate at 100 °C for 3 min and then diluted to 0.5 % sodium dodecyl sulphate. For electrophoresis about 50 μg of the dissolved membrane protein was layered on top of polyacrylamide gels (5 %, ratio bis-acrylamide : acrylamide 0.03). The electrode buffer was 0.5 M sodium phosphate, pH 7.2 with 0.5 % sodium dodecyl sulphate. After 10 min at 1 mA per gel the current density was adjusted to 5 mA per gel and the electrophoresis continued for 4 h. Bromophenol blue was used as the tracking dye. All gels were run in duplicate. One gel of each pair was then stained with Coomassie blue to localize the proteins, the other was sliced transversely into 1–2 mm slices to count the radioactivity. Each slice was left over-night in 1 ml Soluene 350 (Packard) at 55 °C. Scintillation fluid (10 ml Instagel, Packard) was then added, and after thorough mixing, the amount of radioactivity in each gel slice was counted.

Control experiments with ANB-phlorizin showed that the rate of disappearance of the absorption band at 320 nm could be used as a measure of the rate of destruction of the azide. Photolysis of a solution of ANB-phlorizin ($4.26 \cdot 10^{-5}$ M) was completed in about 2 min. The presence of a 4,4'-diacetamidostilbene-2,2'-disulfonic acid (DAS) solution ($2 \cdot 10^{-3}$ M) between the light source and the ANB-phlorizin solution did not affect the rate of photolysis of the ANB-phlorizin, phlorizin ($2 \cdot 10^{-3}$ M) increased the time for complete photolysis by about 2-fold. Since in experiments using ghosts suspensions, 20 min was routinely used as the photolysis time, light absorption by phlorizin is unlikely to exert a major protective effect.

RESULTS AND DISCUSSION

ANB-phlorizin is an effective inhibitor of SO_4^{2-} exchange across the erythrocyte ghost membrane when present in the extracellular medium and protected from light. The inhibition is concentration-dependent, 50 % inhibition being achieved at about $1 \cdot 10^{-5}$ M (Table I). The inhibition is reversible and after three washes the

TABLE I

CONCENTRATION-DEPENDANCE OF REVERSIBLE INHIBITION OF SO_4^{2-} EXCHANGE BY ANB-PHLORIZIN

For experimental details see text.

Inhibitor concentration (M)	% Inhibition of SO_4^{2-} exchange
0	0
$1.0 \cdot 10^{-7}$	1.0
$1.0 \cdot 10^{-6}$	15.0
$5.0 \cdot 10^{-6}$	33.0
$1.0 \cdot 10^{-5}$	47.0
$1.0 \cdot 10^{-4}$	94.0

SO_4^{2-} exchange rate returns to the control rate observed in the absence of inhibitor (Fig. 1). Under similar conditions, phlorizin shows a 50 % inhibition of SO_4^{2-} exchange at $6 \cdot 10^{-4}$ M. Using NB-phlorizin, the structure of which is more similar to that of ANB-phlorizin than is that of phlorizin, 50 % inhibition at $2 \cdot 10^{-5}$ M is achieved.

Like phlorizin, intracellular ANB-phlorizin has no effect on the rate of SO_4^{2-} equilibrium exchange when present at the inner membrane surface (Table II). Studies carried out on the rate of efflux of intracellular tritiated ANB-phlorizin from ghosts revealed that, in the temperature range 0–30 °C, after an immediate release from a

TABLE II

EFFECTS OF INTRACELLULAR AND EXTRACELLULAR PHOTOLYZED ANB-PHLORIZIN ON THE RATE OF SO_4^{2-} EXCHANGE

ANB-phlorizin concentration (10^{-5} M)	Membrane surface	$k \cdot 10^{-3} \cdot min^{-1}$	% inhibition
None	—	9.34	—
1.3, photolyzed	external	6.18	34
1.0, photolyzed	internal	9.53	0
None	—	11.1	—
4.3, photolyzed	external	3.74	66
4.3, photolyzed	internal	11.6	0
None	—	10.9	—
1.3, photolyzed	external	7.24	34
2.86, photolyzed	external	5.62	48
2.15, photolyzed	internal	10.5	4
None	—	9.97	—
0.57, photolyzed	external	9.22	8
1.43, photolyzed	external	7.45	25
2.15, unphotolyzed	external	7.27	27
1.08, unphotolyzed	internal	10.5	0
1.08, photolyzed	internal	10.2	0

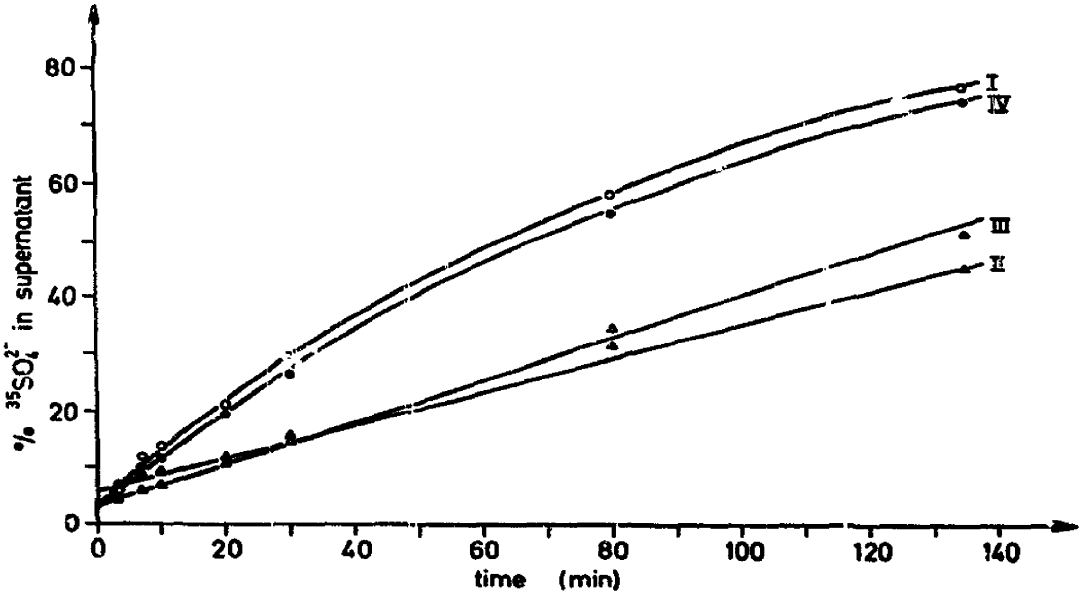

Fig. 1. Time-course of efflux of $^{35}SO_4^{2-}$ from ghosts. For details of experimental procedure see Materials and Methods. I, ghosts photolyzed in buffer; II, ghosts photolyzed in presence of $1.33 \cdot 10^{-5}$ M ANB-phlorizin; III, not photolyzed, $1.0 \cdot 10^{-5}$ M ANB-phlorizin present during SO_4^{2-} efflux measurement; IV, no photolysis, $1.0 \cdot 10^{-5}$ M ANB-phlorizin added to cells and removed by washing prior to determination of $^{35}SO_4$ efflux rate. Ordinate: $^{35}SO_4^{2-}$ present in medium as % of $^{35}SO_4^{2-}$ in medium at infinite time.

small population of leaky ghosts (about 4% of the total population), the residual intracellular agent did not penetrate the membrane in any detectable quantity during 1 h.

Photolysis at approx. 320 nm for 20 min of a 10% suspension of ghosts at 14 °C in a medium containing ANB-phlorizin results in an irreversible inhibition of SO_4^{2-} exchange (Fig. 1). Photolysis for longer periods of time does not alter the extent of inhibition. Comparable concentrations of ANB-phlorizin invariably produced less irreversible inhibition after photolysis than reversible inhibition before photolysis. Experiments were carried out using tritiated ANB-phlorizin in order to correlate the kinetic observations with binding to components of the erythrocyte membrane and to examine the accessibility of the cytoplasmic membrane surface to intracellular reagent. Following photolysis with extracellular ANB-phlorizin the radioactivity was rather evenly distributed throughout the gel region where proteins are located, with a slight peak in the 95 000 molecular weight region and a large rapidly migrating peak beyond the Bromophenol blue front (Fig. 2). A similar rapidly migrating peak has been observed in previous work using photoactivated labels on membrane preparations and it has been suggested that this peak is due to photolyzed or unmodified reagent which is non-covalently bound [5, 6]. We believe that in our experiments this peak represents a covalent attachment of the photolabel to the membrane lipids. This belief is supported by the finding that the peak did not disappear after up to 20 washes of the ghosts prior to electrophoresis. More-

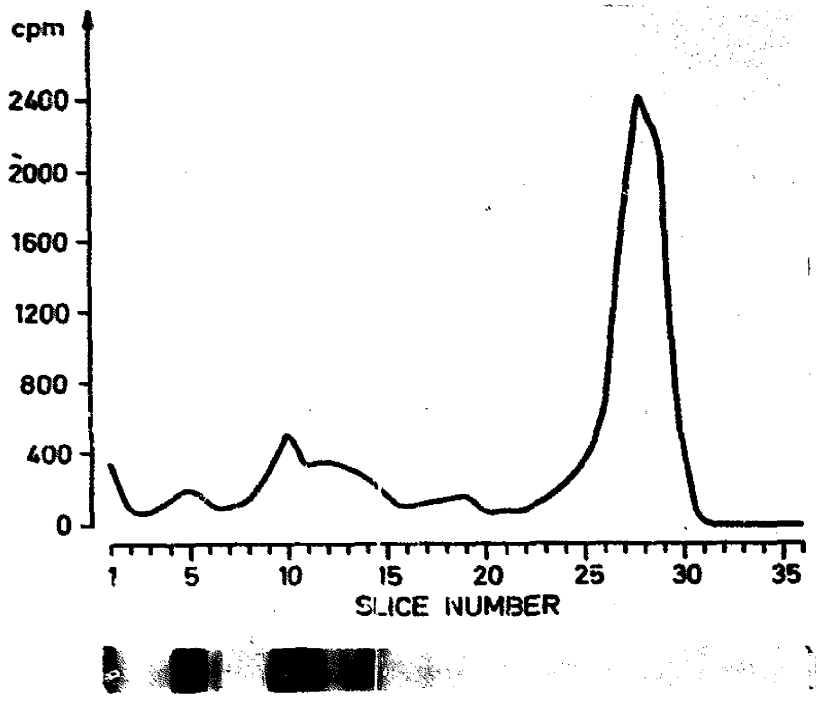

Fig. 2. Distribution of radioactivity in electroferogram of ghosts photolyzed in ANB-phlorizin solution. For details of electroforesis procedure see text. Distribution of radioactivity was determined in duplicate of gel photographed under curve after staining for proteins.

over, if photolysis was omitted, no radioactivity was bound to the ghost membranes. Thin-layer chromatography of chloroform/methanol extracts of lyophylized labelled ghosts revealed a pattern of products similar to that obtained after photolyzing pure phospholipid liposomes in a solution of ANB-phlorizin. Although labelling is absolutely dependent upon radiation and is not affected by repeated washing, ANB-phlorizin that had been photolyzed and then added to ghosts is also firmly bound to the cell membrane constituents which could be separated on the gels. Only in the lipid region is the proportion of this binding small as compared to the binding produced by phlorizin in the presence of cells (Fig. 3).

The concentration-dependance of irreversible inhibition of SO_4^{2-} exchange and the associated fixation of ANB-phlorizin to the membrane after photolysis reveals that whilst labelling increases with increasing concentrations of ANB-phlorizin, the inhibition of SO_4^{2-} exchange reaches a maximum (Fig. 4). The reduction in the extent of inhibition of SO_4^{2-} exchange seen at the higher concentrations of ANB-phlorizin is associated with a lower retention of $^{35}SO_4^{2-}$ by the ghosts after photolysis. This suggests that at high concentrations of ANB-phlorizin a leak is produced during photolysis. This suggestion is supported by the observation that resealed ghosts which contained 140 mM KCl, 20 mM Tris · Cl, pH 7.2 lost their K^+ when irradiated in the presence of ANB-phlorizin, the K^+ loss depending upon the ANB-phlorizin concentration in the irradiated suspension (Table III). Subsequent studies

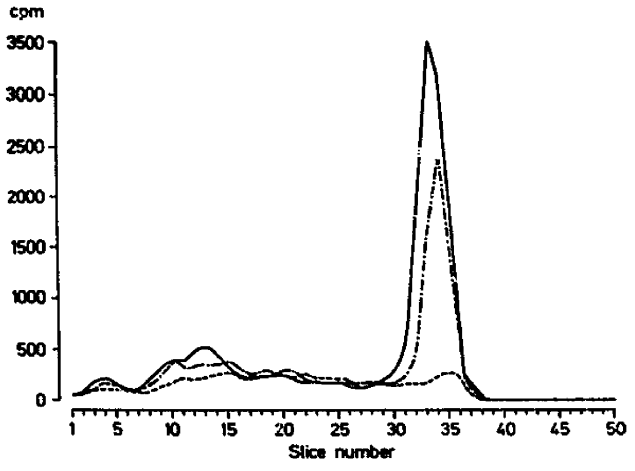

Fig. 3. Distribution of radioactivity in ghost membranes after photolysis of intracellular and extracellular ANB-phlorizin. — and –·–·–, gels from ghosts photolyzed with $1.075 \cdot 10^{-5}$ intracellular ANB-phlorizin and $1.43 \cdot 10^{-5}$ M extracellular ANB-phlorizin; – – –, gel from ghosts added to a solution of $1.43 \cdot 10^{-5}$ M ANB-phlorizin which had been photolyzed before addition of ghosts. Total protein per gel 61.5 μg, 67.5 μg and 65.25 μg, respectively.

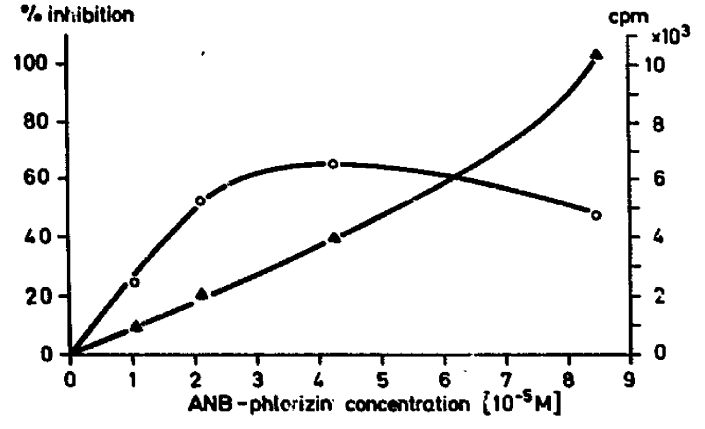

Fig. 4. Concentration-dependance for inhibition of SO_4^{2-} exchange and binding to ghost membrane after photolysis in media containing ANB-phlorizin. Abscissa: concentration of ANB-phlorizin in photolysis medium. Ordinates: left ordinate, % inhibition of SO_4^{2-} exchange relative to unphotolyzed control in absence of ANB-phlorizin; right ordinate, radioactivity in lipid peak, normalized for different protein content of the gels at the various ANB-phlorizin concentrations. ○—○, inhibition data; ▲—▲, binding data.

on the sidedness of the effect of irreversibly bound ANB-phlorizin were limited to the lower concentration range of the dose-response curve in Fig. 4.

The labelling of the ghost membrane after photolysis in the presence of photolysis extracellular or intracellular ANB-phlorizin is shown in Fig. 3. The labelling pattern obtained under these conditions refers to the same ghosts which had been used in the determination of inhibition of SO_4^{2-} exchange presented in Table II (fourth experiment). The distribution of radioactivity after photolysis of the ghosts

TABLE III

EFFECTS OF PHOTOLYSIS IN THE PRESENCE OF ANB-PHLORIZIN ON K^+ RETENTION BY K^+-LOADED GHOSTS

% K^+ retention after photolysis	ANB-phlorizin concentration (M)
3	1.10^{-4}
82	1.10^{-5}
100	1.10^{-6}
100	0

with intracellular and extracellular ANB-phlorizin shows that the agent is able to become covalently attached to the membrane from either surface (Fig. 3)*. Although similar labelling patterns are seen with the intracellular or extracellular ANB-phlorizin, only extracellular binding results in inhibition of SO_4^{2-} transport (Table II).

The present work establishes that the lack of inhibition by the intracellular photo-reactive phlorizin derivative is not due to the inaccessibility of the inner membrane surface. It strongly suggests that the binding sites responsible for inhibition of SO_4^{2-} transport do not reside on a mobile carrier but are confined to the outer membrane surface.

The labelling of membrane components after photolysis is not sufficiently selective to determine the specific site of action involved in the photo-activated inhibition. Since the work was stimulated by observations on the sidedness of action of phlorizin and since the photo-activated inhibition is accomplished using a derivative of phlorizin it was of interest to see whether or not sites involved in the reversible inhibition of SO_4^{2-} exchange by phlorizin were labelled by ANB-phlorizin. The irreversible inhibition of SO_4^{2-} exchange after photolysis of ANB-phlorizin is reduced

TABLE IV

REDUCTION OF IRREVERSIBLE INHIBITION OF SO_4^{2-} DUE TO PRESENCE OF PHLORIZIN IN PHOTOLYSIS MEDIA WITH ANB-PHLORIZIN

ANB-phlorizin concentration (M)	Phlorizin concentration (M)	$k \cdot 10^{-3} \cdot min^{-1}$	% irreversible inhibition of SO_4^{2-} exchange
0	0	8.9	—
$1.60 \cdot 10^{-5}$	0	4.6	48.3
$1.60 \cdot 10^{-5}$	$2.0 \cdot 10^{-3}$	7.0	21.3
$1.60 \cdot 10^{-5}$	$2.0 \cdot 10^{-4}$	5.2	41.6
$1.60 \cdot 10^{-5}$	$2.0 \cdot 10^{-5}$	4.7	47.2
$1.20 \cdot 10^{-5}$*	0	4.8	46.1

* ANB-phlorizin added at start of kinetics, protected from light.

* The lack of significant labelling of spectrin after photolysis of intracellular ANB-phlorizin is similar to the recent finding that spectrin is not labelled using another intracellular photoreactive agent [7].

TABLE V

PROTECTION AGAINST IRREVERSIBLE PHOTO-ACTIVATED INHIBITION OF SO_4^{2-} EXCHANGE DUE TO PRESENCE OF REVERSIBLY ACTING INHIBITORS OF SO_4^{2-} EXCHANGE

ANB-phlorizin concentration $4.26 \cdot 10^{-5}$ M.

Conditions	$k \cdot 10^{-3} \cdot min^{-1}$	% irreversible inhibition of SO_4^{2-} exchange
Control	9.43	—
ANB-phlorizin	3.56	62.2
ANB-phlorizin + $2 \cdot 10^{-4}$ M NB-phlorizin	5.47	42.0
ANB-phlorizin + $2 \cdot 10^{-3}$ M phlorizin	5.32	43.6
ANB-phlorizin + $2 \cdot 10^{-3}$ M DAS	5.75	39.0

by the presence of phlorizin in the irradiated suspension, the protection depending upon the phlorizin concentration in the irradiated suspension (Table IV). Protection is also produced by NB-phlorizin (Table V). However, a similar effect is seen when another reversibly acting inhibitor of SO_4^{2-} exchange, DAS, is present in the irradiated suspension (Table V). The partial protection against irreversible inhibition after photolysis afforded by the presence of the reversibly-acting inhibitors of anion exchange was correlated with a decrease in binding of the radioactive label. No specific protection could be detected of either lipid or protein. Under similar conditions glucose (5 mM) had no detectable effect on either the irreversible inhibition of sulphate exchange or on the binding of the radioactive label to membrane components.

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