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Enhancement of divalent anion transport across the human red blood cell membrane by the water-soluble dansyl chloride derivative 2-(*N*-piperidine)ethylamine-1-naphthyl-5-sulfonylchloride (PENS-Cl)

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Sulfate transport across the red cell membrane is enhanced by the newly synthesised, water-soluble and nonpenetrating dansyl chloride derivative 2-(*N*-piperidine)ethylamine-1-napththyl-5-sulfonylchloride (PENS-Cl). The transport is only enhanced if the potentiating agent 2-(4-aminophenyl-3-sulfonic acid)-6-methylbenzothiazol-7-sulfonic acid (APMB) is present during incubation with PENS-Cl. The enhanced flux is reduced by the anion-transport inhibitor 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate (H_2DIDS) to about the same low level as in untreated controls. In contrast to dansyl chloride, PENS-Cl does not increase cation leakage from the red cells. The effects of PENS-Cl on sulfate transport resemble those produced by dansyl chloride. However, it can be shown that PENS-Cl only reacts with one subset of sites that are modified by dansyl chloride and involved in bringing about the enhancement of sulfate transport. This subset does not include the sites accessible to dansyl chloride in the absence of APMB. It comprises only a fraction of the sites exposed to dansyl chloride in the presence of APMB. Very little labelling of proteins of the red cell membrane can be seen after exposure of ghosts to the PENS-Cl, while dansyl chloride labels all major proteins.

Introduction

The band 3 protein in the human red cell membrane accomplishes transport of both monovalent and divalent inorganic anions. Nevertheless, the pH dependence of the transport processes of the two types of anions differs. This observation has attracted much attention and has led to the hypothesis that in contrast to the monovalent anions, the divalent anions can only be transported together with a proton [1] ('titratable carrier model' [2–4]). This implies that the translocation of both monovalent and divalent anions by the band 3 protein is only feasible when anion binding to the substrate site increases the protein's negative net charge by no more than one unit.

The relationship between monovalent and divalent anion transport has been studied in much detail (for reviews see Refs. 5–8), but only little of the pertinent work involved the use of covalent chemical modification. One exception is the study of the effects of dansylation of the red cell membrane [9], which produces reciprocal effects on chloride and sulfate equilibrium exchange; the

Abbreviations: APMB, 2-(4-aminophenyl-3-sulfonic acid)-6-methylbenzothiazol-7-sulfonic acid; H_2DIDS, 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonate; dansyl chloride, *N*,*N*-dimethyl-1-aminonaphthyl-5-sulfonylchloride; PENS-Cl, 2-(*N*-piperidine)ethylamine-1-naphthyl-5-sulfonylchloride; DNFB, 1-fluoro-2,4-dinitrobenzene; SDS, sodium dodecyl sulfate; DMF, dimethylformamide.

former is slightly inhibited, the latter considerably enhanced [10]. Both effects are potentiated when dansylation is carried out in the presence of the disulfonic acid APMB, an inhibitor of anion transport which acts by combination with the band 3 protein at the same site at which H_2DIDS and related compounds produce inhibition. However, inhibition of chloride equilibrium exchange is much less augmented than the acceleration of SO_4^{2-} transport, indicating that it is possible to dissociate to some extent the effects on transport of the two types of anion species [10,11]. There is reason to believe that in the presence of APMB one or several additional dansyl chloride binding sites become exposed which are not accessible in the absence of APMB, and hence that the acceleration of anion transport is due to the modification of more than one single amino acid residue.

Although the effect on anion transport of dansylation is quite specific, the binding of dansyl chloride to the red blood cell membrane is not. In addition to the haemoglobin, all membrane proteins that can be separated by one-dimensional SDS-polyacrylamide gel electrophoresis, as well as the amino lipids, become fluorescent [17]. This indicates that dansyl chloride penetrates across the red blood cell membrane and finds access to many of the potentially reactive groups such as amino, imidazole, tyrosine and SH-groups [12].

After dansylation, the band 3 protein becomes fluorescently labelled on both the 35 and 60 kDa chymotryptic fragments. This makes it impossible so far to correlate the modification of specific amino acid residues of the transport protein with the observed effect.

In view of the specificity of the actions of dansyl chloride, it seemed useful to synthesize a derivative which is not only specific with respect to the actions on transport but which also reacts more selectively with specific amino acid residues on the band 3 protein. The present paper reports the synthesis of a positively charged dansyl chloride derivative, PENS-Cl, and shows that it produces similar changes of divalent anion transport as dansyl chloride. The derivative does not penetrate and, although in the test-tube it reacts with amino, histidine and sulfhydryl groups, it modifies the various membrane constituents with a much higher selectivity. In contrast to dansyl chloride, the compound produces little if any increase of cation permeability. In addition to its increased specificity of binding and action, the compound has the advantage that, in contrast to dansyl chloride, it is easily soluble in water.

Materials and Methods

Synthesis of PENS-Cl (Fig. 1) A solution of 7 g 1-hydroxynaphthylsulfonic acid (I) (0.031 mol; a gift of Bayer AG) and 8.99 g *N*-(2-aminoethyl)piperidine (II) (10 ml, 0.07 mol; EGA Chemie) in 150 ml 25% Na_2SO_3 was titrated with concentrated HCl to pH 8. The brown solution was refluxed for 72 h. During that time, a white precipitate appeared. After cooling to room temperature, the pH was adjusted to 6 and the solution stored overnight on ice. The precipitate was collected by suction and washed with ice-cold water. For purification, the precipitate was dissolved in water by adding sufficient 50% NaOH to obtain a pH of about 12 and refluxed in the presence of charcoal for 2 h. After filtration over silica, PENS (III) was precipitated from the clear solution by adding 12 M HCl to pH 6 and cooling for several hours on ice. It was collected on a sintered glass filter and dried in vacuo over P_2O_5. The yield was about 80%. On TLC (Kieselgel;

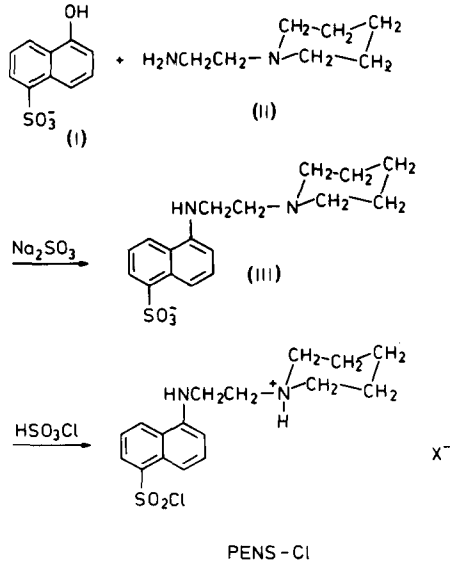

Fig. 1. Synthesis of PENS-Cl. For explanation see text.

methanol/ acetone/ water (60 : 30 : 10, v/v)), PENS-OH moved as a single spot ($R_F = 0.62$) which yielded blue fluorescence during illumination with ultraviolet light (366 nm) (adduct: $R_F = 0.89$).

To convert PENS into PENS-Cl, 1 g of the dry PENS (III) was dissolved in 10 ml HSO_3Cl and kept for 72 h under N_2 at room temperature in a closed flask. This solution was added dropwise to a vigorously stirred mixture of 15 g $NaHCO_3$ in dry methanol on an ice-bath. After filtration, the methanol was removed from the yellow solution by evaporation. For further purification, the PENS-Cl was dissolved in a small volume of methanol and filtrated over Sephadex LH-20. The methanol was removed as described above and the PENS-Cl was freeze-dried. The ^{1}H-NMR spectra were consistent with the proposed structure and showed no impurities. The signals for the aromatic system were at (relative to TMS in $C^2H_3O^2H$) $\delta = 8.6$ (1 H), 8.2 (2 H), 7.6 (2 H) and 6.9 (1 H); for the aliphatic system, 3.2–3.9 and 1.8–2.0 (14 H). On TLC (Kieselgel; acetone), PENS-Cl showed a single yellow spot with R_F of about 0.5, while dansyl chloride moved at the solvent front.

The reaction product between PENS-Cl and 2-aminoethanol and dansyl chloride and 2-aminoethanol were purified by preparative TLC (2 mm Kieselgel; methanol). The fluorescence data (excitation and emission) are presented in Table I.

PENS-Cl is easily soluble in methanol, DMF and, with decomposition, in water; it is less soluble in acetone, ethanol and other nonpolar solvents.

Using TLC (Kieselgel; methanol/ acetone (60 : 40, v/v), PENS-Cl could be shown to react in aqueous solutions with the following compounds: NH_3, imidazole, N^α-acetyllysine, N^α-acetylcysteine, 2-aminoethanol and histidine-glycine; whereby, in the latter compound, both the glycine-NH_2 and the histidine-imidazole were modified. No reaction with N^α-acetyltyrosine ethyl ester was observed.

Preparation of red blood cell ghosts and flux measurements. All experiments were performed with red cell ghosts prepared from human red cells (Rh^+O) by methods described previously [13]. Except in the experiment described in the legend to Fig. 5, the ghosts were dansylated in 'standard medium' containing 1 mM Na_2SO_4, 130 mM NaCl, 20 mM EDTA (pH 6.6) with or without APMB, at the concentrations indicated in figures or tables. The temperature was 20 or 37°C; under the conditions described, the effect on transport as measured at 30°C was independent of the temperature during dansylation. PENS-Cl was added as a methanolic solution to give final concentrations of PENS-Cl and methanol of 250 μM and 1% (v/v), respectively. The controls received the same methanol concentrations without PENS-Cl. The reaction was carried out at 20°C in the dark for 45 min. At the end of the reaction period, the ghosts were washed once in the standard medium, which was adjusted to pH 8.0 and contained 0.1 mg/ml bovine serum albumin, and two more times in the same medium without albumin. The sulfate equilibrium exchange was measured at pH 8.0, 30°C, by determining the efflux of $^{35}SO_4^{2-}$. The details of the flux measurements and their evaluation were essentially similar as described by Legrum et al. [9]. SDS-polyacrylamide gel electrophoresis was performed on 5–20% linear acrylamide gradient slab gels (180 × 160 × 0.8 mm; stacking gel 3.5% acrylamide) using the buffer system of Laemmli [14].

TABLE I

COMPARISON OF FLUORESCENCE PROPERTIES OF DANSYL AND PENS DERIVATIVES

Exitation (λ_{ex}) and emission (λ_{em}) wavelengths in methanol. Relative intensities are related to dansyl-OH (100%). Wavelengths as read from the instrument without corrections. The maximum of exitation for PENS-aminoethanol was very broad and showed in addition to the peak at 384 nm, two lower peaks at 350 and 397 nm.

Compound	λ_{ex} (nm)	λ_{em} (nm)	Relative intensity (%)
Dansyl-OH	331	468	100
PENS	350	475	80
Dansyl-aminoethanol	347	522	40
PENS-aminoethanol	384	520	25

Results and Discussion

In contrast to dansyl chloride, PENS-Cl produces little if any increase of net cation movements across the red blood cell membrane. (Fig.

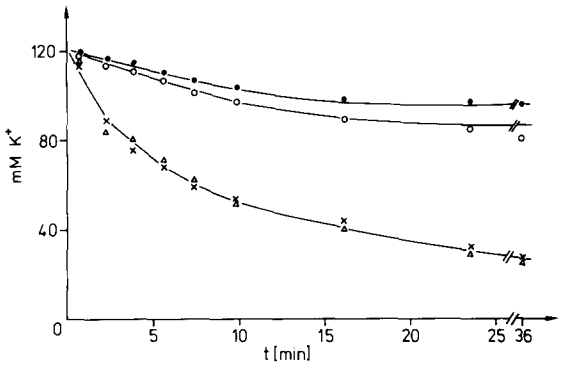

Fig. 2. Time-course of K^+ loss from ghosts which had been incubated without (●) or with 250 μM PENS-Cl plus 5 mM APMB for 45 min (○), or with 125 μM dansyl chloride with or without 5 mM APMB, respectively (×, △), for 30 min. The medium inside and outside the ghosts had the same composition and consisted of 20 mM EDTA, 130 mM KCl. To measure K^+ loss, the ghosts were resuspended in a medium that contained NaCl in place of KCl. Net K^+ efflux was measured at pH 8.0, 30°C, by flame photometry. Ordinate: K^+ concentration inside the ghosts. Abscissa: time in min.

2). The equilibrium exchange of sulfate is slightly, but probably not significantly enhanced (Table II). Approx. 30-fold enhancement is seen, however, when the exposure to PENS-Cl is performed in the presence of the potentiating agent APMB. The effects of PENS-Cl are smaller than those seen with dansyl chloride, but evidently much more specific with respect to potentiation by APMB. Under our experimental conditions, repetition of the exposure to dansyl chloride or PENS-Cl does not increase further the effects on SO_4^{2-} transport. This applies to repeated modification both in the presence or absence of APMB.

The enhanced sulfate transport in the modified red cell ghost is inhibited by H_2DIDS, an agent that is known to react with a one-to-one stoichiometry with the band 3 protein (Table II). This indicates that the enhancements described refer to band 3-mediated equilibrium exchange and not to leaks produced by the modification reagents.

The response of SO_4^{2-}-equilibrium exchange to modification with PENS-Cl in the presence of APMB shows essentially the same features as the response after exposure to dansyl chloride. Most important, as previously observed after dansylation in the presence or absence of APMB, after treatment with PENS-Cl plus APMB, the pH dependence of sulfate transport in the modified ghosts no longer shows the maximum around pH 6 that is typical for the untreated red cell ghosts (Fig. 3). This maximum is supposed to be the result of the superimposition of an acceleration of SO_4^{2-} transport with decreasing pH (due to the increased availability of H^+ for SO_4^{2-}/H^+ cotransport) and an inhibition (due to the increased occupancy of an inhibitory modifier side) [4]. After treatment with PENS-Cl, increasing the pH does no longer reduce the rate of SO_4^{2-} transport. Instead, the SO_4^{2-} transport steadily increases until a plateau is reached, which resembles

TABLE II

COMPARISON OF EFFECTS OF DANSYL CHLORIDE AND PENS-Cl ON SULFATE EQUILIBRIUM EXCHANGE

Rate constants for sulfate efflux $^{o}k_S$ from untreated ghosts, and ghosts incubated with 125 μM dansyl chloride or with 250 μM PENS-Cl, each with or without 5 mM APMB in a medium containing 130 mM NaCl, 20 mM EDTA and 1 mM Na_2SO_4 (pH 6.6). Sulfate equilibrium exchange was measured after removal of APMB and the unreacted or hydrolyzed dansyl derivatives at 30°C, pH 8.0, in the medium described above. H_2DIDS concentration was 10 μM. The averages refer to six independent determinations, n.s., not significant.

	Rate constant $^{o}k_S$ (min^{-1}) ($\times 10^3$)				
	Control	PENS-Cl	PENS-Cl + APMB	Dansyl chloride	Dansyl chloride + APMB
no H_2DIDS	3.8 ± 0.5	8.7 ± 1.1	99.0 ± 6.0	62.0 ± 8.0	319 ± 40
+ H_2DIDS	1.0 ± 0.3	1.7 ± 0.2	2.7 ± 0.2	2.4 ± 0.7	2.8 ± 1.0
Acceleration of H_2DIDS-sensitive transport	–	n.s.	34	21	113

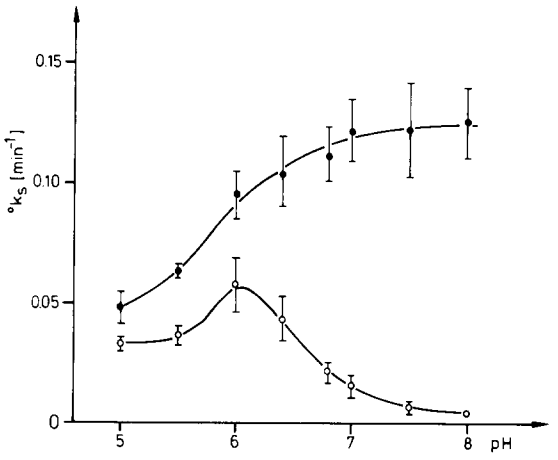

Fig. 3. pH dependence of sulfate equilibrium exchange. Ghosts were incubated with 250 μM PENS-Cl plus 5 mM APMB for 45 min. Sulfate equilibrium exchange was measured after three washes to remove PENS-Cl and APMB at 30°C at the pHs indicated on the abscissa. Ordinate: $^{o}k_S$ (min^{-1}). H_2DIDS-insensitive flux is subtracted in all measurements. ●, Ghosts incubated with PENS-Cl; ○, untreated ghosts. Bars represent standard errors of the mean of four independent experiments.

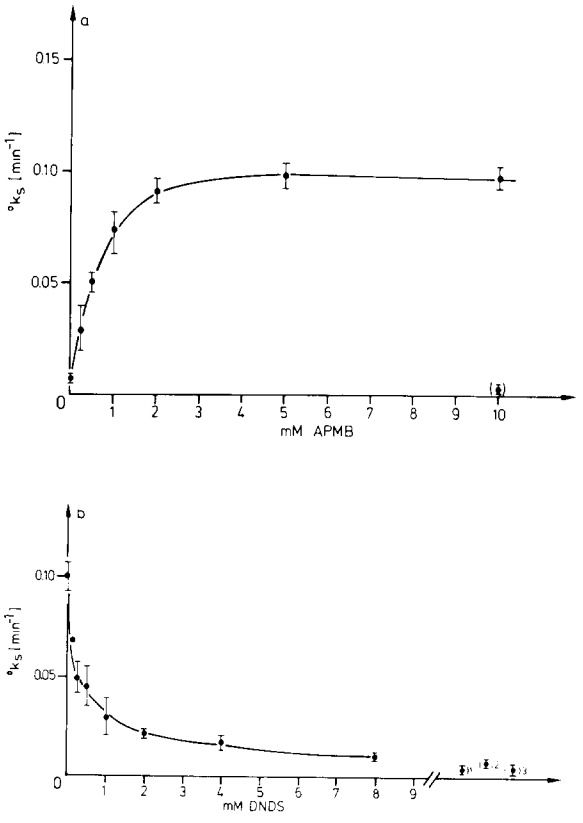

Fig. 4. (a) Effect of varying APMB concentration during incubation with 250 μM PENS-Cl for 45 min. Mean values from three experiments. The single data point refers to a control without PENS-Cl. Abscissa: APMB concentration. The corresponding H_2DIDS-insensitive fluxes are subtracted from all values. (b) Effect of DNDS on modification of sulfate equilibrium exchange during incubation with 250 μM PENS-Cl plus 5 mM APMB for 45 min. Abscissa: DNDS concentration. Ordinate: $^{o}k_S$ (min^{-1}). Sulfate equilibrium exchange was measured at 30°C, after terminating the modification reaction by three washes in the absence of PENS-Cl, APMB and DNDS. The single data points refer to controls without APMB, DNDS and PENS-Cl (1), cells incubated with PENS-Cl only (2) and cells incubated with PENS-Cl in the presence of 8 mM DNDS (3).

that seen with monovalent anions at low temperatures [15].

Other features that are characteristic for the modification of red cell ghosts with dansyl chloride were also seen after treatment with PENS-Cl. The potentiation by APMB is saturable and reaches a plateau at about 5 mM (Fig. 4a). The effect of a fixed concentration of 5 mM APMB is counteracted by the presence of increasing concentrations of DNDS (Fig. 4b), a stilbenedisulfonic acid that also reacts with the H_2DIDS-binding site, but apparently stabilizes a conformational state of that site that is different from a conformational state stabilized by APMB [10]. Finally, like dansylation, treatment with PENS-Cl produces the more acceleration of sulfate equilibrium exchange the higher the Cl^- concentration during exposure to the agent (Fig. 5), suggesting that there exist allosteric interactions between a substrate-binding site and the amino acid residues which are susceptible to reaction with PENS-Cl.

Previous studies suggested that there exist at least two distinct classes of dansyl chloride binding sites in the red cell membrane that are involved in bringing about the observed changes of the kinetics of sulfate equilibrium exchange. One class is accessible to dansylation regardless of whether or not APMB is present, the other only when APMB is present [10]. The former class of sites seems to be accessible only to dansyl chloride but not to PENS-Cl. The latter class seems to be available to both dansyl chloride and PENS-Cl, although not all of the sites involved in dansyl chloride binding are also susceptible to PENS-Cl.

The evidence for these conclusions is sum-

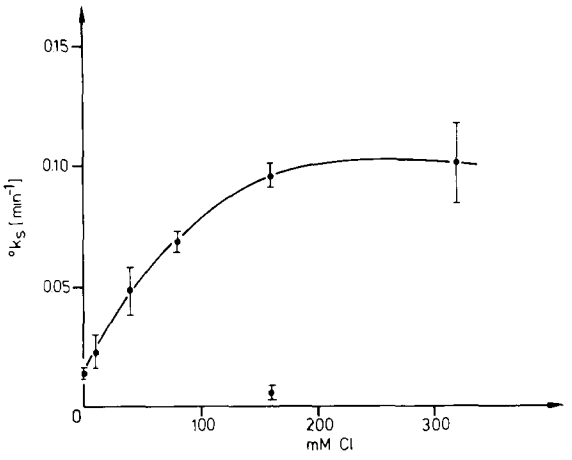

Fig. 5. Effect of varying Cl^- concentration during incubation with 250 μM PENS-Cl in the presence of 5 mM APMB. The ghosts were resealed in media containing 20 mM EDTA, 1 mM Na_2SO_4 and KCl at the concentrations indicated on the abscissa. After incubation with PENS-Cl at the KCl concentrations indicated for 45 min, the ghosts were washed in their respective suspension medium to remove breakdown products and APMB. Regardless of the KCl concentration at which incubation with PENS-Cl had been carried out, they were then equilibrated for 60 min in identical media containing 130 mM KCl and 0.2 μM valinomycin in addition to 20 mM EDTA and 1 mM Na_2SO_4. This leads to the establishment of equal Cl(i)− concentrations in the ghosts that had received their treatment in solutions of different Cl^- concentrations. Thereafter, the valinomycin was removed by one wash in a solution containing 20 mM EDTA, 130 mM KCl, 1 mM Na_2SO_4 and 0.5% bovine serum albumin and two more washes in the same medium without albumin. Sulfate equilibrium exchange was then measured at 30°C, pH 8.0, in the medium described at the fixed KCl concentration of 130 mM. Ordinate: $^{o}k_S$ (min^{-1}). The single data point pertains to ghosts which had been incubated in the absence of PENS-Cl and APMB. H_2DIDS-insensitive flux is subtracted in all measurements.

marized in Table III. The table shows that dansylation in the absence of APMB produces an approx. 15-fold increase of sulfate equilibrium exchange (column II, row (1)), while PENS-Cl produces an increase of about 1.5-fold, which is prob-

TABLE III

EFFECT OF CONSECUTIVE TREATMENTS WITH DANSYL CHLORIDE AND PENS-Cl

Rate constants for sulfate efflux $^{o}k_S$ ($\cdot 10^3$ min^{-1}) at 30°C, pH 8.0, in 130 mM NaCl, 20 mM EDTA and 1 mM Na_2SO_4. For the experiments shown in the table, ghosts were made from erythrocytes which had been obtained from the blood bank after removal of leukocytes and serum (packed erythrocytes). In contrast to ghosts prepared from erythrocytes which had been stored together with their serum and leukocytes, in ghosts made from packed erythrocytes, treatment with PENS-Cl in the presence of APMB enhances the sulfate equilibrium exchange only about 10-times (instead of about 30-times as seen in erythrocytes stored in their own plasma). Ghosts were incubated for a first time with the reagents given under '1st incubation period', then after removal of breakdown products and APMB, for a second time with the compounds given under '2nd incubation period'. Conditions of incubations were the same as described in Table II. Standard error of the mean of four independent experiments. In brackets: enhancement of SO_4^{2-} exchange compared to untreated controls. The H_2DIDS-insensitive flux is not subtracted.

	I	II	III	IV	V
1st incubation period:	Control	Dansyl chloride	Dansyl chloride + APMB	PENS-Cl	PENS-Cl + APMB
2nd incubation period					
(1) No further treatment	3.02 ± 0.65 (n.s.)	43.18 ± 13.3 (14.3)	315.0 ± 93.22 (104.3)	4.40 ± 1.26 (n.s.)	37.9 ± 4.27 (12.6)
(2) Dansyl chloride	–	–	–	44.6 ± 10.41 (14.8)	100.44 ± 25.14 (33.3)
(3) Dansyl chloride + APMB	–	–	–	172.75 ± 38.27 (57.2)	216.5 ± 41.62 (71.7)
(4) PENS-Cl	–	36.9 ± 6.47 (12.2)	238.8 ± 81.18 (79.1)	–	33.8 ± 1.85 (11.2)
(5) PENS-Cl + APMB	–	63.2 ± 8.43 (20.9)	267.8 ± 55.12 (88.7)	18.5 ± 2.14 (6.1)	–

ably not significant (IV, (1)). Moreover, treatment of the red blood cell ghosts with PENS-Cl prior to exposure to dansyl chloride does not affect the dansyl chloride-induced increase of sulfate exchange (compare IV, (2) to II, (1)). PENS-Cl applied after dansylation leads to a slight, probably insignificant, decrease of the effect of the previous dansylation (II, (4)). Thus, the enhancement of sulfate equilibrium exchange by dansyl chloride is independent of treatment with PENS-Cl prior or after dansylation. A plausible explanation of this finding would be that the nonpenetrating PENS-Cl cannot reach the binding sites for the easily penetrating dansyl chloride. This would imply that the binding sites for dansyl chloride are located either in the hydrophobic region of the membrane or at the inner membrane surface.

At least some of the additional binding sites for dansyl chloride which become accessible when dansylation is carried out in the presence of APMB are also accessible for PENS-Cl. This is suggested by the observation that exposure to PENS-Cl in the presence of APMB induces at least 9-fold acceleration of SO_4^{2-} equilibrium exchange, as compared to exposure in the absence of APMB (IV, (1) and V, (1)). This is much less than the increment of SO_4^{2-} exchange after dansylation in the presence of APMB (II, (1) and III, (1)). If the cells are first treated with APMB plus PENS-Cl and subsequently with APMB plus dansyl chloride, then a further increase of sulfate equilibrium exchange is observed (V, (3)). This increase is considerably larger than the sum of the increments seen after exposure to APMB plus PENS-Cl and subsequent exposure to dansyl chloride in the absence of APMB (V, (2)). This indicates that at least part of the increment is due to dansylation of APMB-exposed binding sites. On the other hand, the total effect of exposure to PENS-Cl plus APMB and subsequent treatment with dansyl chloride plus APMB (V, (3)) is smaller than the effect of dansyl chloride plus APMB alone (III, (1)). This would suggest that some of the binding sites exposed by APMB had been occupied by the less effective PENS-Cl prior to reaction with dansyl chloride.

The results would suggest that APMB exposes two classes of binding sites: one which is accessible to dansyl chloride alone and another that is accessible to both dansyl chloride and PENS-Cl *. The latter should reside on the outer membrane surface since, as may be recalled, PENS-Cl does not penetrate (see below).

As shown above, a specific enhancement of sulfate equilibrium exchange that is a characteristic consequence of the modification of the anion-transport system by dansyl chloride is also observed after treatment with PENS-Cl. Nevertheless, the labelling of membrane constituents is difficult to detect and the labelling pattern is different. Although dansyl chloride and PENS-Cl fluoresce with the same intensity, very little labelling can be seen with the latter, under conditions where the compound produces the described changes of anion transport and where dansyl chloride would label all proteins and lipids normally detectable by gel electrophoresis [17] (Fig. 6, see lanes 1, 2, 4, 6, 8). Moreover, no penetration of the compound could be detected since bovine serum albumin, incorporated into the ghosts, does not become fluorescent (see lanes 3, 5, 7, 9), although it easily reacts when exposed to PENS-Cl in free solution. The higher selectivity of the compound is related to the fact that it does not penetrate. When white, freely permeable ghosts, prepared by the method of Dodge et al. [16] were used, membrane proteins were labelled to a similar extent as with dansyl chloride (see Fig. 6, lane 10). Thus, PENS-Cl seems to represent a modification reagent which reacts only at the outside of the red cell membrane. The labelling of band 3 in resealed ghosts is too weak to make it suitable for localization within the peptide chain of the band 3 protein by fluorescence measurements of the modified amino acid residues responsible for the enhancement of diva-

* There is one detail in Table III that requires an additional comment. When ghost that had first been modified with PENS-Cl (IV, (1)) or subjected to an additional treatment with PENS-Cl plus APBM, sulfate transport is enhanced (IV, (5)). The transport rate does not, however, reach the same high value that is seen after modification with PENS-Cl in the presence of APMB without preceding treatment with PENS-Cl (V, (1)). This result is also obtained when ghosts that had first been treated with PENS-Cl plus APMB (V, (1)) are subsequently exposed to PENS-Cl (V, (4)). One gains the impression that without affecting anion transport, during the repeated exposure of the ghosts to PENS-Cl the potentiating action of APMB is reduced.

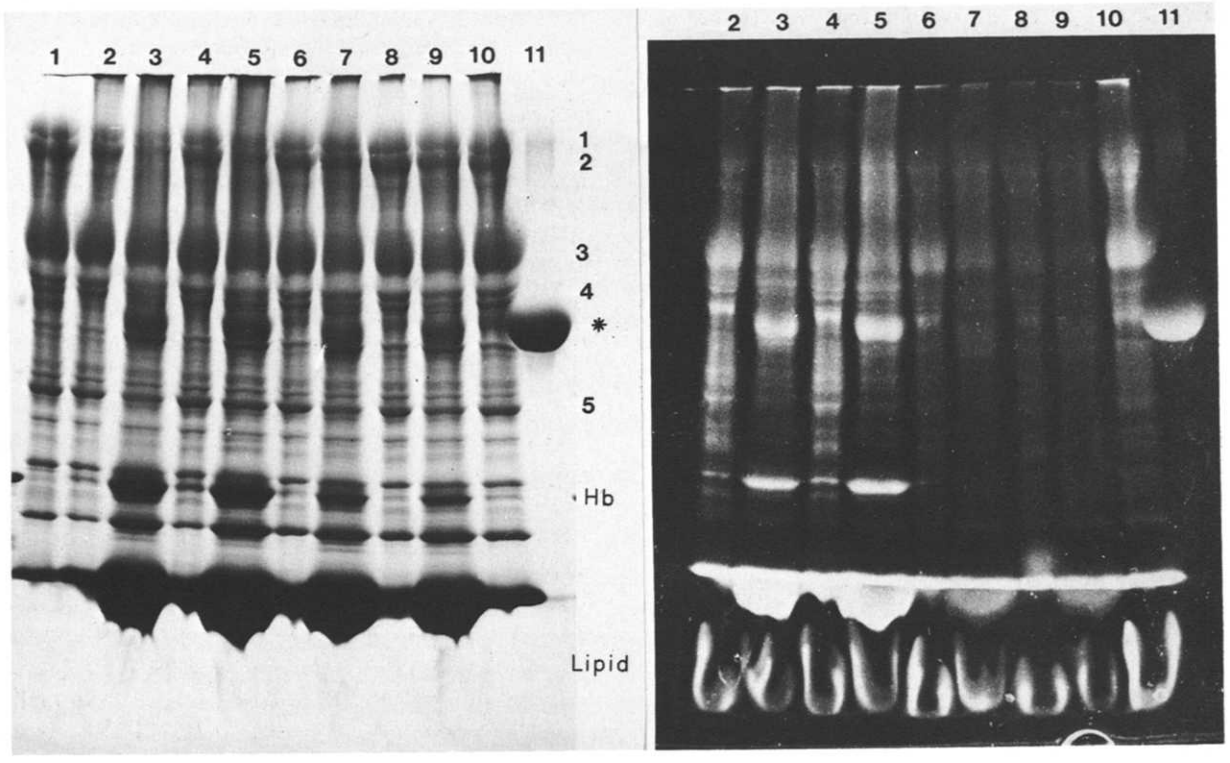

Fig. 6. Gel electrophoretogram (5–20% linear acrylamide gradient; 3.5% stacking gel) of ghosts (if bovine albumin was incorporated) or stroma incubated with dansyl chloride or PENS-Cl in the presence or absence of APMB. (1) No treatment; (2) incubated with dansyl chloride; (3) as (2) but with 0.1% albumin inside; (4) incubated with dansyl chloride in the presence of 5 mM APMB; (5) as (4) with 0.1% albumin inside; (6) incubated with PENS-Cl; (7) as (6) with 0.1% albumin inside; (8) incubated with PENS-Cl in the presence of 5 mM APMB; (9) as (8) plus 0.1% albumin inside; (10) ghosts after hemolysis with 5 mM sodium phosphate (pH 8.0) incubated with PENS-Cl; (11) albumin incubated with PENS-Cl. The gel to the right had been illuminated with ultraviolet light (366 nm) prior to Coomassie blue staining, shown to the left. Overloading of the gel with protein was necessary for detection of the minor fluorescent bands. Nomenclature according to Steck [18]. Hb = Hemoglobin, ★ = bovine albumin.

lent anion transport. Thus, the compound needs to be synthesised in a radioactively labelled form to make it useful for this purpose.

Perhaps it may be useful to add that like dansyl chloride, PENS-Cl does not react with the outward-facing lysine residue *a* on the 17 kDa segment of the band 3 protein. This residue is allosterically linked to the substrate binding site. When modified by reaction with DNFB or suitable isothiocyanates, anion transport is inhibited [19]. In the experiments with dansyl chloride, the survival of that lysine for reactions with DNFB and H_2DIDS was shown [9]. In the experiments described in this paper, lysine *a* was protected against modification with PENS-Cl by the presence of APMB, which binds reversibly near this lysine residue and prevents it from getting dinitrophenylated. Modification with PENS-Cl in the absence of APMB neither inhibits anion transport nor the capacity of the band 3 protein to combine with H_2DIDS or APMB (Tables II and III).

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