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THE REACTION OF 1-DIMETHYLAMINONAPHTHALENE-5-SULFONYL CHLORIDE (DANSCl) WITH ERYTHROCYTE MEMBRANES

A NEW LOOK AT "VECTORIAL" MEMBRANE PROBES

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SUMMARY

1. Dansylation of intact human and sheep erythrocytes, as well as ghosts derived therefrom, has been employed to study the reactivity of the plasma membrane proteins of intact cells and membranes isolated therefrom. Dansylation was monitored by fluorescence and by use of 1-[^{3}H]dimethylaminonaphthalene-5-sulfonyl chloride ([^{3}H]DANSCl).

2. For this, a new, gentle dansylation method was developed, the fluorochrome being dispersed ultrasonically into lecithin–cholesterol micelles, suspended in an isoosmotic, aqueous medium in which intact cells could be reacted for 120 min without damage. DANSCl in aqueous solution and not incorporated into micelles rapidly forms DANSOH, which does not label the erythrocyte membranes.

3. With excess DANSCl added to intact cells the membrane proteins bind about 64% of the reagent, the remainder combining with hemoglobin. However, the amount of DANS bound/mg hemoglobin is only $7 \cdot 10^{-4}$ that of membrane proteins.

4. After dansylation of intact cells or ghosts the membrane peptides were separated by electrophoretic molecular sieving in polyacrylamide, laden with sodium dodecyl sulfate.

5. In the case of isolated erythrocyte ghosts, apparently all membrane peptides detectable by other stains are accessible to the label. When intact cells are used DANSCl reacts predominantly with a few peptides, which appear identical with those reacting with labels considered impermeant. In the case of human erythrocytes a peptide of apparent mol. wt 90000 and a presumed glycopeptide with apparent mol. wt about 60000 react most vigorously. In the case of sheep erythrocytes, three components are labelled unusually prominently; they have apparent mol. wts of about 155000, 95000 and 40000; the last may be a glycopeptide.

6. Of the lipids, only phosphatidylethanolamine appears to become dansylated

Abbreviation: DANSCl, 1-dimethylaminonaphthalene-5-sulfonyl chloride.

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to a measurable extent, but more than 90% of this phosphatide remains unreacted. However, a strongly dansylated, unidentified compound migrates with the lipids.

7. Our data, and a critical review of other labelling procedures, suggest the divergent labelling of the proteins of intact cells' membranes and ghosts cannot be attributed unambiguously to the leakiness of the latter, and may also reflect a change n the reactivity of membrane proteins produced by membrane isolation.

INTRODUCTION

The gross permeability properties of erythrocyte membranes tend to resemble those of a thin lipid layer. This could come about if large areas of these cells' plasma membranes were in fact purely lipid, or if the membrane were a lipid–protein mosaic[1]. In either situation the size and charge of the permeating molecule would influence its rate of penetration and, in the case of a mosaic, its dimensions and other properties might also limit diffusion of relatively large lipophilic molecules. Because of the lipomimetic properties of biomembranes and their relative impermeability to large anions, several workers have synthesized special anionic labels presumed to react almost exclusively with components exposed at the external surface of intact erythrocytes, but at both surfaces of isolated erythrocyte membranes[2]. We are not convinced that any of these low-molecular weight reagents are truly vectorially specific and suspect that they may reveal not only membrane "sidedness" but also reactivity changes in the membrane proteins of intact cells and membrane isolates.

We have accordingly employed a different approach, using a highly lipophilic reagent, 1-dimethylaminonaphthalene-5-sulfonyl chloride (DANSCl) to label membrane proteins in intact erythrocytes and membranes isolated thereform. In principle this reagent should either react equivalently with all proteins of intact erythrocytes, or preferentially with those on the interior of the postulated lipophilic membrane; the latter because of the high energy required to transfer such an apolar molecule into the aqueous cytoplasmic phase and to the hemoglobin "sink" therein. In fact, however, we find that DANSCl reacts with the proteins of intact erythrocyte membranes and isolated ghosts in a manner analogous to highly charged or otherwise impermeant labels. This circumstance, suggests that, while permeability changes could contribute to the differential labelling of membrane proteins in intact cells *vs.* membrane isolates, alterations of the reactivity of the membrane proteins during isolation may be as or more important.

The use of water-insoluble reagents such as DANSCl is complicated by the fact that addition of the reagents in the organic solvents usually employed to dissolve the dye[3–5] invariably perturbs the membranes to the point of lysis. Moreover, we have not been able to dansylate membrane components detectably using very low levels of DANSCl in aqueous buffers, presumably because of its well-known, rapid conversion to nonreactive DANSOH[4]. We have accordingly devised a new labelling procedure in which the dye is first incorporated into cholesterol–lecithin micelles in aqueous dispersion, whence it reacts with accessible groups on the membrane. In this approach about 20% of the dye is in free form as non-reactive DANSOH. The bulk of the reactive species incorporates into the relatively large micellar particles.

MATERIALS AND METHODS

Unless stated otherwise, all materials were of analytical grade. We obtained DANSCl, A grade, from Fluka AG; synthetic L-dipalmitoyllecithin (A grade, chromatographically homogeneous) from Calbiochem and pure crystalline cholesterol from Fluka AG. [^{3}H]DANSCl was obtained from Radiochemical Centre Amersham (TRK 384; 250 μCi in 0.25 ml benzene, 5.1 Ci/mmole).

Human (O, Rh^+) or sheep erythrocytes (acid–citrate–dextrose blood), both not older than 28 days, were freed of leukocytes and platelets by 5-fold differential centrifugation in 0.15 M NaCl, 0.005 M sodium phosphate, pH 8.0, at 4 °C. Suspensions of intact erythrocytes were adjusted to A 0.68 at 546 nm[6] with isoosmolar, phosphate-buffered sucrose (52 mM Na_2HPO_4, 77 mM NaH_2PO_4, 155 mM sucrose), pH 9.0. Erythrocyte ghosts were prepared as described[7].

Lecithin, cholesterol and DANSCl (75.0:33.8:7.5, by wt) were dissolved in 5 ml chloroform–methanol (2:1, v/v), 3 drops of water added and the organic solvents removed *in vacuo* at about 0 °C. The yellow, hydrated residue was then taken up in 15 ml phosphate–sucrose, pH 9.0, and sonicated for 9 min at 0 °C (Branson Sonifier, Micro Tip, power step 1). The resulting suspension was centrifuged at $100000 \times g_{max}$ for 30 min (Beckman, Spinco L265B centrifuge, rotor 50.1) to remove large aggregates. The slightly opalescent, greenish-yellow supernatant was used to dansylate intact erythrocytes or membrane fragments therefrom.

The equilibrium between soluble and micellarized reagent was determined with [^{3}H]DANSCl using gel permeation chromatography on Bio Gel P_4, 50–150 mesh (Bio-Rad Laboratories) and ultrafiltration (Diaflo Ultrafiltration membrane, Type PM10, Amicon Corp.). In each case the lipids were extracted after fractionation as in ref. 8 and analysed by quantitative thin-layer chromatography[9].

For labelling we mixed 12 ml of the micellar dispersion with 10 ml of human or sheep erythrocytes and let the reaction proceed for up to 180 min at 4 °C. Ghosts from an equivalent number of erythrocytes were treated identically. Thereafter intact cells were washed four times with 40 ml phosphate–sucrose, pH 8.0, prior to preparing ghosts as in ref. 7. Dansylated ghosts were washed four times in 5 mM phosphate, pH 8.0. For electrophoresis, the ghosts were dissolved in 1% sodium dodecyl sulfate, 40 mM dithiothreitol[10,11] and the membrane proteins separated in 4.15% polyacrylamide gels (diameter 6 and 10 mm, for 105 min, at currents of 5 or 20 mA/gel, respectively), using 40 mM Tris–20 mM acetate–2 mM EDTA (pH 7.4) as buffer. The 6 mm gels, ultimately stained with Coomassie blue, were loaded with 40 μg; the larger gels can be loaded with up to 500 μg. Dissolved membrane proteins were quantified by the ninhydrin reaction[12] and spectrofluorometrically[13]. We used fuchsin as tracker dye. Coomassie blue or periodate–Schiff staining was as described in refs 10 and 11. The gels were scanned at 620 nm for Coomassie blue and 560 nm for periodate–Schiff staining, using a Gilford spectrophotometer (Model 240) with a synchronized scanner (Model 2410-5).

The 10-mm gels containing dansylated membrane peptides were photographed using a Polaroid MP-3 camera, with a Schott GG 455 nm filter, Polaroid Land film Type P/N (50 ASA), Desaga UVIS universal irradiator and Desaga Intensive UV-Source (366 nm) as light sources; the exposure was for 240 s at f-stop 8. Under these conditions the degree of film blackening varied logarithmically with the concentration

of fluorophore under observation. The Polaroid negatives were scanned in the Gilford scanner to compare the dansylation pattern with staining patterns obtained using Coomassie blue and periodate–Schiff reagent. The scanner readings were recalculated to correct for the fact that the instrument measures absorbance which is a log function of intensity, while the blackening of the photographic negatives is already logarithmic.

Fluorescence spectra (uncorrected) were measured with the Hitachi Perkin–Elmer fluorescence spectrophotometer (Model MPF-2A). For native peptide fluorescence excitation was at 286 nm and emission was read at 340 nm, with bandwidths of 15 nm and 20 nm, respectively. For DANS fluorescence, excitation was at 328 nm and emission was measured at 495 nm, using the same respective bandwidths. Our instrument has also been adapted to scan fluorescence in polyacrylamide gels (by mechanically advancing them past the incident beam), but this approach is still generally inferior to the photographic technique. Nonetheless, this approach has allowed us to establish the dansylation kinetics of certain membrane peptides.

Membrane lipids were extracted[8], and separated by thin-layer chromatography on Kieselgel H containing 0.5% carboxymethyl-cellulose[9], using chloroform–methanol–water (6:4:1, by vol.) as solvent system. Dansylation of membrane lipids was determined fluorimetrically after extraction of lipids separated by thin-layer chromatography. DANS-phosphatidylethanolamine (R_F 0.768) and the native phosphatide (R_F 0.718) were quantified according to phosphate content[14].

To determine the degree of hemoglobin reaction during dansylation of intact erythrocytes, these were lysed[7] and particulate material removed by centrifugation at $100000 \times g_{av}$ for 60 min (Spinco ultracentrifuge L265B, rotor 50.1, 4 °C). The supernatant was then used to purify hemoglobin–globin[15] and its degree of dansylation compared with that of ghosts from erythrocytes dansylated according to our methods. Dansylated globin was freed of unbound DANSCl by 3-fold extraction with chloroform–methanol (2:1, v/v).

Micellar dispersions containing [^{3}H]DANSCl were prepared as described above but 50 μl benzene containing [^{3}H]DANSCl (7 μCi) were added to the unlabelled DANSCl–lipid mixture before removing organic solvents *in vacuo*. [^{3}H]DANSCl binding to ghosts and hemoglobin was determined after 180 min reaction. For counting, samples were diluted 10-fold in distilled water, after addition of a few drops of H_2O_2, solubilized in Aquasol (Packard) and counted in a Packard Tricarb Scintillation Counter (Model 3320).

RESULTS

Excitation and emission spectra of DANSCl

The important parameters of the excitation and emission spectra are given in Table I. The emission maximum of the dye in micellar suspension is surprisingly close to that found in dilute aqueous solutions. The excitation spectrum of DANS combined with membrane protein shows an additional excitation maximum at 290 nm, due to energy transfer from tyrosine–tryptophan to the bound extrinsic fluorophore.

Effects of dansylation on erythrocytes and erythrocyte ghosts

Erythrocytes retain their normal osmotic properties during reaction with

micellar DANSCl for up to 12 h. Also, sodium dodecyl sulfate electrophoresis followed by staining with Coomassie blue shows no significant differences between the peptide pattern of the membranes from normal non-dansylated erythrocytes (Tables IIA and IIB).

The osmotic stability of the dansylated erythrocytes and the nearly identical electrophoretic distribution of membrane protein — measured with Coomassie blue — before and after dansylation of intact erythrocytes (Tables II), suggests that our labelling method is a rather gently one for intact cells. However, dansylation of isolated ghosts changes their peptide distribution pattern, causing the appearance of seemingly aggregated components, O_{1-2} (apparent mol. wt) >300000), which we assume to arise from peptides of normally lower molecular weight. They cannot be split by heating to 100 °C for 30 min in sodium dodecyl sulfate, 40 mM dithiothreitol. The effect may be due to non-specific aggregation. However, one should

TABLE I

EXCITATION (λ_{max}^{exc}) AND EMISSION MAXIMA (λ_{max}^{em}) OF DANSCl

Conditions	λ_{max}^{exc} *(nm)*	λ_{max}^{em} *(nm)*
1. In phosphate (5 mM) pH 8	321	505
2. In 96% ethanol	315	472
3. Micellar DANS-lecithin–cholesterol suspension	323	500
4. DANS-membrane peptide	290	
	328	492

TABLE IIA

Quantitative molecular weight distribution of human and sheep erythrocyte membrane peptides under various conditions on the basis of Coomassie blue staining. The proportion of the various peptides is given as the percentage of the total.

Peptide	*Apparent mol. wt*	*Proportion of Coomassie blue staining using*		
		Ghosts from normal human erythrocytes	*Ghosts from dansylated human erythrocytes*	*Dansylated human erythrocyte ghosts*
1	300 000	17.0	16.5	13.7
2	270 000	14.4	14.9	11.5
2_1	250 000	4.0	2.6	2.5
3	90 000	27.5	27.4	24.7
4 A, B	77 000	9.3	9.3	8.9
	71 000			
$4_{1,2}$	58 000	8.6	9.5	10.0
	50 000			
5	42 000	4.9	4.8	3.7
6	37 000	3.0	3.8	4.1
6_1	30 000	4.1	3.6	3.8
$0_{1,2}$	>300 000	—	—	12.3

TABLE IIB

Peptide	*Apparent mol. wt*	*Proportion of Coomassie blue staining using*		
		Ghosts from normal sheep erythrocytes	*Ghosts from dansylated sheep erythrocytes*	*Dansylated sheep erythrocyte ghosts*
1	290 000	12.8	11.9	10.0
2	260 000	12.9	12.9	10.4
2_1	245 000	4.0	3.8	4.0
3	155 000	8.9	9.4	4.6
4	130 000	4.5	4.6	4.9
5	95 000	25.3	25.5	22.2
6 A, B	80 000	7.4	7.1	6.5
	75 000			
$6_{1,2,3}$	69 000	6.9	6.9	7.7
	57 000			
7	42 000	6.4	6.4	6.5
8, $8_{1,2,3}$	38 000	7.1	9.5	6.6
	23 000			
$0_{1,2}$	> 300 000	—	—	13.5

also consider covalent coupling of membrane peptides which are closely apposed in the membrane, but it is unclear how this should come about during dansylation. Possibly some of the reactions described by Mildenstein[16] are involved. We are investigating this matter further.

Reaction of DANSCl with erythrocytes and erythrocyte ghosts

We have used [^{3}H]DANSCl in addition to the unlabelled reagent to achieve the highest possible sensitivity for binding studies. Both approaches to separate free DANSCl from the micellar form gave essentially identical values. By gel filtration 79% of the DANSCl appeared with lipids in the exclusion volume and 74% of the

TABLE III

REACTION OF DANSCl WITH THE MEMBRANES AND HEMOGLOBIN OF HUMAN AND SHEEP ERYTHROCYTES

A representative experiment. Total activity is that of the micellar suspension used for dansylation. The loss of radioactivity into the sediment corresponds to the bound DANSCl. The supernatant after cell lysis is considered to reflect hemoglobin-bound DANS.

Erythrocytes	*Total radio-activity (cpm)*	*Residual radio-activity after 180 min reaction and cell sedimentation (cpm)*	*Supernatant after cell Lysis and 100000 × g*		*Ghosts*	
			Total	*cpm/mg protein*	*Total*	*cpm/mg protein*
Human	171 200	162 639	3234	7.9	5904	11 354
Sheep	163 450	152 777	3885	9.6	6788	13 054

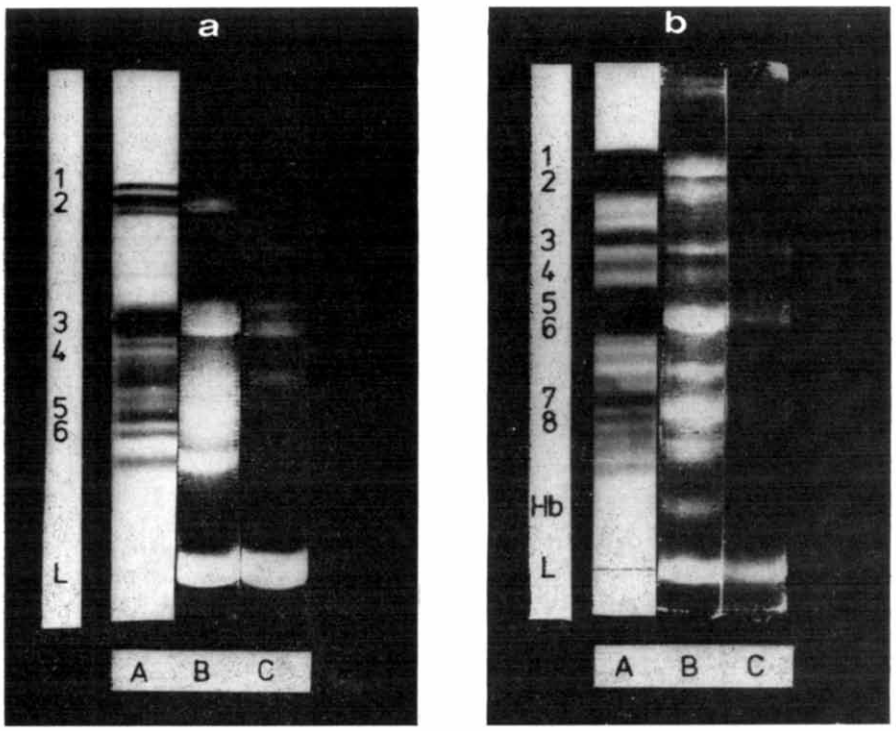

Fig. 1. Dansylated membrane peptides from human (a) and sheep (b) erythrocytes (Gel C) and from treated erythrocyte ghosts (Gel B). The peptides are numbered as described in refs 10 and 11, the lowest number corresponding to the highest molecular weight (see also Figs 2a and 2b and Table II. Bands $O_{1,2}$ have a mol. wt > 300000 and appear upon dansylation of human and sheep erythrocyte ghosts. For comparison Coomassie blue stained electropherograms of untreated human and sheep erythrocyte ghosts are shown on the left of each panel (Gel A).

reagent was retained by ultrafiltration. The ratio of "free" to micellar DANSCl does not change after incubation with erythrocytes or erythrocyte ghosts.

The "free" component does not react with erythrocytes or their membranes even to an extent detectable by radioactivity measurements and labelling depends fully upon incorporation of the dye into the lipid micelles.

The binding of DANSCl to the membranes and hemoglobin of intact human and sheep erythrocytes is given in Table III. In this we assume that [^{3}H]DANSCl binds identically to unlabelled DANSCl. Human erythrocytes bind 5.35% of the added DANSCl, 64% of which is recovered in the isolated membranes. In the case of sheep erythrocytes, 6.5% of the added DANSCl is bound, of which 63% is recovered in isolated membranes. In both species hemoglobin binds only 0.07% as much DANS/mg protein as the membrane proteins.

The distinctive reaction of DANSCl with membrane peptides of intact erythrocytes and of erythrocyte ghosts is illustrated in Figs 1 and 2. These represent electrophoretic separations of membrane proteins/peptides in 4.15% polyacrylamide containing 1% sodium dodecyl sulfate, and compare the DANS-binding of ghosts derived from dansylated intact sheep and human erythrocytes with that of sheep

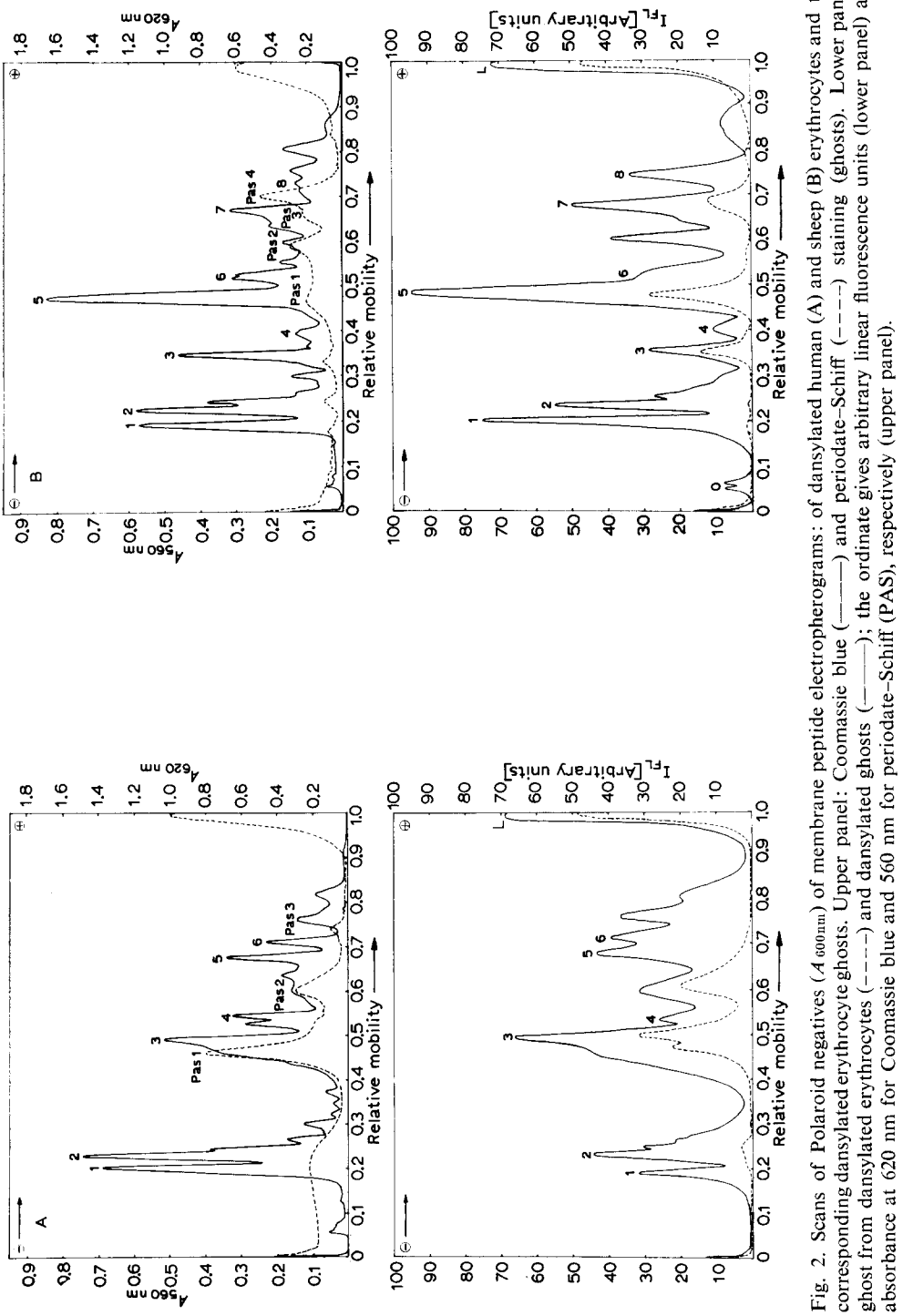

Fig. 2. Scans of Polaroid negatives (A_{600nm}) of membrane peptide electropherograms: of dansylated human (A) and sheep (B) erythrocytes and the corresponding dansylated erythrocyte ghosts. Upper panel: Coomassie blue (———) and periodate–Schiff (– – – –) staining (ghosts). Lower panel: ghost from dansylated erythrocytes (– – – –) and dansylated ghosts (———); the ordinate gives arbitrary linear fluorescence units (lower panel) and absorbance at 620 nm for Coomassie blue and 560 nm for periodate–Schiff (PAS), respectively (upper panel).

and human erythrocyte ghosts dansylated after isolation. Replicate gels were also stained with Coomassie blue and periodate–Schiff. Protein/peptide bands are numbered as described in refs 10 and 11, the lowest number corresponding to the highest molecular weight.

In ghosts all the major membrane peptides recognizable by Coomassie or periodate–Schiff staining are accessible to DANSCl. However, not all components react equivalently with the various stains. Thus Component 5 stains relatively more vigorously with DANSCl than with Coomassie blue, and the reverse is true for Components 1 and 2. This is not unexpected since neither staining method is absolute. In contrast, the reaction of the fluorochrome is quite restricted when intact cells are dansylated: First the specific labelling of those components that do react is less intense than in ghosts, by a factor of 3. Second, in the case of intact cells, only a few membrane peptides react unambiguously. In the case of human erythrocytes (Fig. 2A), DANSCl reacts primarily with Peptide(s) 3 (apparent mol. wt 90000)

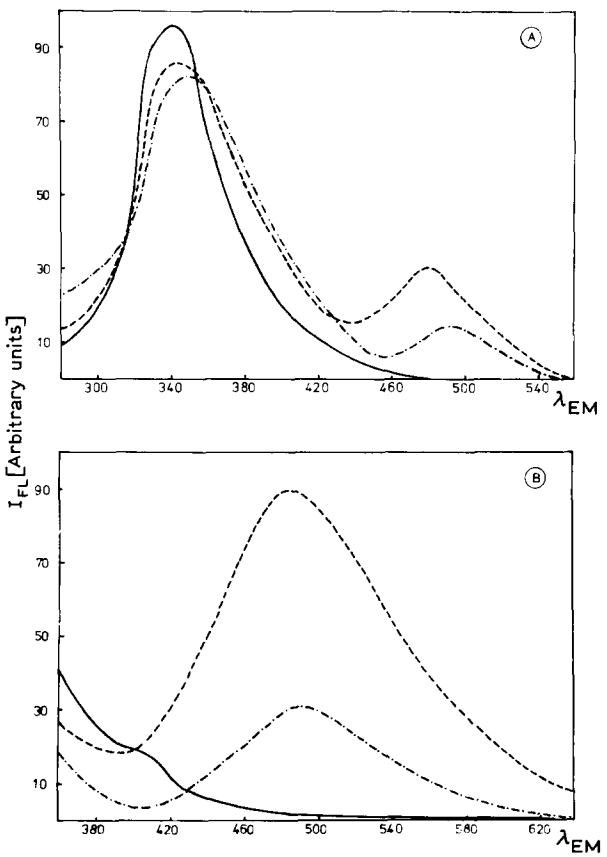

Fig. 3. Emission spectra of membrane peptides derived from dansylated erythrocytes (60 μg/ml) –•–•–, DANS-globin prepared from dansylated hemoglobin (40 μg/ml) – – – –, and globin prepared from hemoglobin of dansylated erythrocytes (40 μg/ml) ———. The excitation wavelengths are 286 nm (a) and 335 nm (b), respectively. Fluorescence intensity is in arbitrary linear units. All measurements were done in 5 mM phosphate, pH 8.0.

and a somewhat slower moving component, which is only partly resolved, as well as a component(s) (apparent mol. wt 60000) corresponding to glycopeptide periodate–Schiff 2. Trace labelling sometimes occurs in the region of Peptide 2. In the case of sheep erythrocytes (Fig. 2B), DANSCl combines primarily with Peptides 3 and 5 *plus* another component with somewhat higher mobility than Peptide 7 (apparent mol. wt near 43000); periodate–Schiff staining suggests that the last might be a glycoprotein (Fig. 2B). Peptides 1 and 2 label occasionally, as illustrated, but not consistently. We cannot exclude trace reactions with other membrane peptides any more than can be done with other labels.

Importantly, while the hemoglobin of dansylated erythrocytes binds about $7 \cdot 10^{-4}$ the amount of DANS/mg protein combined with membrane proteins, this amounts to about 35% of the DANS reacting with intact cells, because of the preponderance of hemoglobin. The disproportionately large specific labelling of those membrane proteins which combine in intact cells may have no vectorial significance, rather indicating their greater reactivity. In any event, the amount of hemoglobin labelling suggests that there is ample opportunity for all membrane peptides to be dansylated during the dye's diffusion to the interior, provided they are reactive (Figs 3A and 3B).

Kinetics of the membrane peptide dansylation

The dansylation kinetics (Fig. 4) of membrane Peptides 3 and 5 intact sheep erythrocytes were determined in polyacrylamide gels. When we measure the ratios of dansyl fluorescence (excitation 328 nm, emission 495 nm) to protein fluorescence (excitation 286 nm, emission 340 nm) for these components at varying times after exposure of intact cells to DANSCl, we find that 60% of the peptide is labelled already within 5 min. The ratio rises from 0.2 after 5 min to 0.38 after 120 min in Peptide 5; Peptide 3 behaves similary. The fluorescence of the low molecular weight component becomes apparent only after 30–60 min.

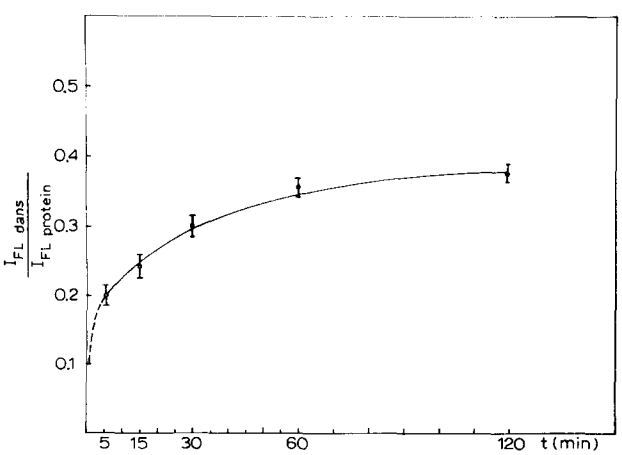

Fig. 4. Time course of dansylation of membrane peptide 5 of sheep erythrocytes. The DANS- and protein fluorescence of the band were measured in the gel. The ordinate gives the ratio [DANS fluorescence/tryptophan fluorescence].

Dansylation of membrane lipids

Of the membrane lipids, only phosphatidylethanolamine and phosphatidylserine are potentially capable of reacting, but both in dansylated erythrocyte and dansylated ghosts, less than 10% of the former is labelled and we have not been able to detect the latter. Disruption of the membranes does not increase this yield, probably because of the NH_2 group's high pK[17] and the vicinity of the negatively charged phosphate. The fluorescence intensity at the front cannot be explained by lipid labelling only and may arise from dansylated amino acids, small peptides or even larger ones with anomalous mobilities.

DISCUSSION

Assuming the same binding characteristics for DANSCl and [^{3}H]DANSCl about $5 \cdot 10^8$ molecules of fluorochrome react per erythrocyte ghosts, provided the DANSCl is presented in a micellar vehicle. DANS in aqueous solution does not yield significant labelling, even when we use highly labelled [^{3}H]DANSCl for detection. However, both our chromatographic and ultrafiltration experiments indicate that 20–25% of the DANS is not in micellar form. We therefore suspect that this fraction is actually DANSOH, which is quite water soluble, readily formed under our conditions[16] but is non-reactive. The micelles may therefore function to (a) concentrate the reactive sulfonyl chloride form at the membrane surfaces, and/or (b) alter the membranes to facilitate reaction with the DANSCl.

It is of great interest that a lipophilic reagent such as DANSCl, should produce the same labelling pattern in intact cells and isolated membranes therefrom, respectively, as is found in systems which are thought to be non-permeant by virtue of hydrophilicity or size[20–24]. Thus, in the case of intact human erythrocytes, two peptides with apparent mol. wts 90000 and one with a mol. wt of about 60000 react most prominently with DANSCl. Correlation of dansylation with periodate–Schiff or Coomassie blue staining of the same gel suggests that the 60000 component is a glycopeptide. In intact sheep erythrocytes DANSCl labels three proteins/peptides preferentially, one of apparent mol. wt about 155000, one of about 95000 and a component with an apparent mol. wt of about 40000, which may be a glycopeptide.

As is true with other labels[18–24], all peptides of isolated membranes which stain with Coomassie blue also label with DANSCl and here too one is tempted to explain this finding by assuming that, while the intact cells' membranes are impermeable to the labels, ghost preparation generates perforations large enough for the reagents to reach the internal membrane surface. However, it appears that under our conditions free DANSCl is quickly converted to non-reactive DANSOH. The fact that hemoglobin, despite its preponderance amounts for only 35% of the total labelling must be ascribed to the lipophilicity of DANSCl, which would tend to retard its diffusion from the membrane phase. This consequence should promote the peptide labelling of intact cells' membranes beyond the extent observed. Hence, since DANSCl clearly lacks vectorial specificity and can label an intracellular component in intact cells, the selective labelling observed when whole cells are treated, indicates that the membrane peptides are differently reactive to DANSCl in intact cells and in membrane isolates thereof.

In this connection it is useful to re-evaluate some of the labelling methods

now frequently used in the analysis of peptide disposition perpendicular to the membrane plane. It would appear that iodination with lactoperoxidase[18,19] is vectorially specific labelling only externally exposed peptides. However, it seems unlikely that reagents such as [^{35}S]sulfanilic acid diazotate[22,23], formylmethionylsulfone phosphate[20,21] and trinitrobenzene sulfonate[25–27] share this property any more than DANSCl. All small molecules of this type indubitably behave as described by Arrotti and Garvin[27] and involve three compartments, the source, a permeation barrier (erythrocyte membrane) and sinks, within the membrane or cell interior, predominantly hemoglobin whose reactive groups combine with the label in question. Labelling of membrane components by a permeating derivativizing compound may occur externally in the vicinity of the source, in transit through the membrane and internally at the cytoplasmic sink. The extent of reaction will depend upon the intrinsic reactivity of the groups involved, the effective concentration of the label at the reactive site and the accessibility of the reactive site. In many cases[27] the concentration of the reagent may fall appreciably during the reaction, affecting all reaction parameters. This is not the case for our experiments with DANSCl, whose concentration dropped by no more than 6.5% in 180 min, but this information is not available for some of the other "vectorial" reagents listed.

Thus, [^{35}S]sulfanilate diazotate[22] is stated to label less than 2% of hemoglobin compared with membrane proteins during 30 min of reaction, but it is unclear whether this value refers to total or specific activity. Certainly within 1 h significant hemolysis occurs. In our experience with this reagent, hemoglobin labelling was considerable within 1 h (unpublished results).

Bretscher's reagent, formylmethionylsulfone phosphate, is used at the unphysiologic pH of 10 (refs 20 and 21). Apart from this, hemoglobin accounts for approximately 15% of the total labelling of erythrocytes during the brief (10 min) reaction period, giving a specific activity of $2.5 \cdot 10^{-3}$ that of the membrane proteins, compared with our value of $7 \cdot 10^{-4}$ for DANSCl after 180 min.

Finally, although Bonsall and Hunt[25] and Steck[26] argue that 2,4,6-trinitrobenzene sulfonic acid reacts preferentially with the externally exposed components of erythrocyte membranes, Arrotti and Garvin[27] clearly show that this compound reacts within the membrane while in diffusion transit to the hemoglobin sink in the cell interior.

We therefore question that the small chemical labels employed to date behave as vectorial monitors exclusively. Their permeability properties indubitably influence their reaction pattern, but not knowing this variable we cannot say that the differential labelling observed in the membrane proteins of intact ghosts has only vectorial meaning. We suggest that the differences may reflect (a) sidedness; (b) alteration of the reactivity of membrane proteins during isolation and/or (c) the lack of the hemoglobin "sink". Without it, many "less reactive" proteins may bind due to label concentration not possible in the presence of the protein. One reviewer suggested another attractive possibility, namely that the native membrane may bear a hydrophilic barrier at the outside, obstructing label transfer, whereas membrane isolates expose large areas where exchange of DANSCl from the membrane can take place.

All labelling studies with small molecules must in our view include explanations other than vectorial labelling in interpretations of the different reactions of the membra-

ne proteins of intact cells and membrane isolates. In particular, one must consider the possibility that in the intact cell the membrane is in its proper physiological state with most of its proteins compactly folded and/or packed, leaving few reactive groups exposed; membrane isolation, however, with its attendent elimination of possible structure determining substances (ions, ATP, hemoglobin), leaves the membrane "relaxed" with many reactive groups available. Of course, permeability changes may appear concurrently.

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