SELECTIVE SOLUBILIZATION OF PROTEINS FROM RED BLOOD CELL MEMBRANES BY PROTEIN PERTURBANTS

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Isolated human erythrocyte membranes were exposed to a series of reagents known to modify or perturb proteins; these included sodium hydroxide, lithium diiodosalicylate, acid anhydrides, and organic mercurials. Each reagent liberated the same set of relatively polar polypeptides from the membrane, while the other, more hydrophobic species invariably remained associated with the membrane residue. The selective elution pattern was precisely that seen previously with 6 M guanidine hydrochloride. The released polypeptides, comprising half of the membrane protein mass, contained no carbohydrate; current evidence indicates that all of these components are confined to the cytoplasmic surface of the membrane. The residue contained all the lipids and all the glycoproteins. The latter are accessible to the outer membrane surface and, in at least two cases, seem to extend asymmetrically across the thickness of the membrane. Thus, the distinctive elution behavior which defines these two groups of polypeptides relates both to their chemical composition and their organizational disposition in the membrane.

INTRODUCTION

The potent dissociating and denaturing properties of sodium dodecyl sulfate (SDS) have fostered the total solubilization and high resolution electrophoretic analysis of membrane polypeptides (cf 1, 2). Many other reagents of more restricted action have been employed to partially disassemble membranes, with the preferential release of one component or another. Such selectivity reflects heterogeneity in the mode of association of various proteins with membranes and thus affords clues to specificity in membrane organization and assembly (2).

Previous studies on isolated erythrocyte membranes have shown that certain polypeptides, numbered **1**, **2**, and **5** herein (3), are co-ordinately released when ionic strength, proton and divalent cation concentrations are reduced (4–6). In contrast, raising the ionic strength above approximately 0.1 selectively releases band **6** (6, 7). All of these polypeptides and several others are preferentially solubilized by treating ghosts with 6 M guanidine-HCl, while bands **3** and **7**, the glycoproteins, and certain minor components remain associated with the membranous residue (8). Recently, 1 mM p-chloromercuribenzene sulfonate (pCMBS) was found to effect the same selective elution pattern as guanidine-HCl (9). In contrast, a variety of other agents, such as n-butanol (cf 10), Triton X-100 (11), succinic anhydride (12), EDTA (13), and even guanidine-HCl (14) have been reported to effect general dissolution of the erythrocyte membrane.

It seemed important to us to distinguish among reagents which principally perturb protein conformation (e.g., denaturants), those selectively disrupting hydrophobic associations (e.g., nonionic detergents), and those acting upon both protein and lipid structure (e.g., alkyl sulfates, such as SDS). In the present study, we analyzed the effects of protein perturbants on isolated human erythrocyte membranes under conditions which did not destroy gross membrane structure. The differential elution pattern observed delineates two groups of polypeptides which are distinguished not only by their ease of dissociation, but also by their glycosylation and their orientation in the membrane (15). In the accompanying report (16), we show that nonionic detergents effect a selective elution profile nearly reciprocal to that described here.

METHODS

Materials

Reagents for polyacrylamide gel electrophoresis were basically those of Fairbanks et al. (6). Inorganic salts and organic solvents were obtained from Fisher, Baker, or Mallinkrodt, succinic anhydride and lithium 3, 5-diiodosalicylate (LIS) from Eastman, and all other reagents from Sigma Chemical Co.

Elution Reactions

Hemoglobin-free human erythrocyte membranes (ghosts) were prepared according to Fairbanks, et al. (6) from fresh or out-dated bank blood in 5 mM Na phosphate, pH 8.0 (5P8). Unless indicated otherwise, one volume of packed ghosts was incubated with 7 volumes of reagent solution, with time, temperature, and pH specified. The supernatant and pellet fractions were then separated by centrifugation at 15,000 rpm for 30 minutes in a Sorvall SS-34 rotor.

Polyacrylamide Gel Electrophoresis

The general protocol was as described by Fairbanks, et al. (6) with these modifications: (i) The gels contained 5.0% acrylamide (recrystallized from hot chloroform) plus 0.19% N,N′–methylenebisacrylamide and 0.2% sodium dodecyl sulfate; the electrode buffer was also 0.2% in SDS (all weight/volume). (ii) The gels were formed under a deionized water overlay and were used after 2–24 hours. (iii) Coomassie blue was used at 0.025%. The trace of stain previously added to the "destaining" solutions for gels containing 1% SDS (6) was omitted. (iv) For periodic acid-Schiff (PAS) staining, overnight fixation in 25% isopropanol–10% acetic acid was sufficient preparation. Exposure to Schiff reagent was for only 1–2 hours. "Aging" the Schiff reagent at 5° for at least a week before use enhanced color development considerably. The gels were destained overnight in 0.1% $Na_2S_2O_5$ –0.01 N HCl and scanned directly the next day. The variable gradient of background absorption originally observed during densitometry (6) was eliminated by filling the scanning cuvet with the acidic $Na_2S_2O_5$ solution instead of water.

Each gel was loaded with protein derived from 10 μl of packed ghosts. For unextracted membranes, this amounts to 34–40 μg protein [6–7 × 10^6 ghosts/μl and 5.7 × 10^{-7} μg protein/ghost (6)]. The major Coomassie blue-stained bands were enumerated according to increasing mobility, as described (3, 6). In this study, the poorly resolved complex of components which migrated between bands **4.2** and **5** and was not eluted by the perturbants was provisionally designated as zone **4.5** (Fig. 1). The 3 principal Schiff-

positive glycoprotein peaks were called PAS 1–3, as before (6; Fig. 5). The tracking dye (TD) position was recorded by an inked needle stab.

Assays

Protein was determined according to the method of Lowry et al. (17), using crystalline bovine serum albumin as a standard. LIS was removed by dialysis before protein analysis. Phospholipid phosphorus was determined following extraction with 2:1 chloroform: methanol (18) and ashing, as outlined by Ames (19). Sialic acid was assayed according to Warren (20) following hydrolysis at 80° for one hour in 0.1 N H_2SO_4. A molar extinction coefficient of 57,000 at 549 nm was verified using N-acetylneuraminic acid as standard. Neutral sugars were estimated by the phenol -H_2SO_4 method (21), using galactose as a standard.

RESULTS

Reagents and experimental conditions were sought which would perturb protein (but not lipid) structure so as to cause the total release of susceptible polypeptide species without destroying the residual membrane. First, we will consider the fate of those polypeptides stained by Coomassie blue. The heavily glycosylated polypeptides are not well visualized by such protein stains (6, 22) and have been analyzed separately by densitometry of PAS-stained gels (see below).

Extraction with Alkali

Reduction in ionic strength and increase in pH of ghost suspensions should differentially release those polypeptides most susceptible to coulombic repulsions. Previous studies (4–6) have, in fact, demonstrated the selective elution of bands **1, 2** and **5** in mildly alkaline buffers of low ionic strength and divalent cation concentration (see also Fig. 3A). Raising the pH to 10 at low ionic strength (Fig. 1A and B) eluted bands **1, 2** and **5,** as expected, as well as band **6** and some undesignated trace components. At pH 11, bands **2.1** and **4.2** became elutable, and at pH 12, band **4.1** was released (Fig. 1C and D). The membranous residue contained bands **3, 7,** and the poorly stained zone designated as **4.5.** Treatment with 0.1 N NaOH caused no additional protein release (compare Fig. 1D and E). The trace of 2-mercaptoethanol used here was not essential to the selective solubilization. At pH $\leqslant$ 12, however, reduced ionic strength was required for effective elution.

Exposure to dilute alkaline media (as with the other reagents discussed below) caused the ghosts to vesiculate extensively (see also refs. 4 and 23). In contrast, extraction with 0.1 N NaOH did not discernibly alter the over-all size and shape of the ghosts in the phase contrast microscope.

The chemical composition of the 0.1 N NaOH extract and residue is given in Table I. Half of the protein but no neutral sugar, sialic acid, or lipid phosphorus was released (despite the potential for alkaline hydrolysis of the phospholipids). Although the membrane protein was evenly divided between the eluted and noneluted fractions, the discerning eye will observe that the NaOH supernatant polypeptides are more heavily stained by Coomassie blue than those retained in the pellet (Fig. 1E). By quantitative densitometry, in fact, the eluted components accounted for approximately 60% of the integrated stain intensity and the retained fraction, only about 40%. This discrepancy

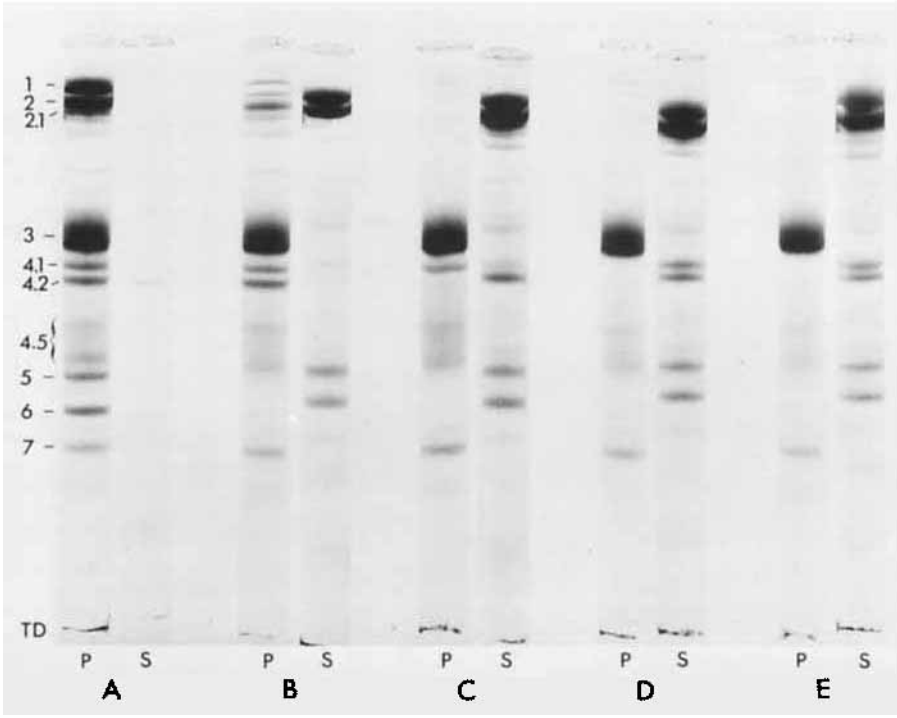

Fig. 1. The release of membrane polypeptides at alkaline pH. One volume of packed ghosts was diluted with seven volumes of ice-cold (A) 5P8, (B–D) water, or (E) 0.1 N NaOH-0.001 M 2-mercaptoethanol. Samples B–D were adjusted with NaOH to pH 10.0, 11.0 and 12.0, respectively, using a low Na^+ error pH electrode. The suspensions were immediately centrifuged. Pellet (P) and supernatant fractions (S) were directly sampled for electrophoresis without neutralization.

Table I. The Chemical Composition of NaOH and LIS Extracts and Residues

	NaOH[a]			LIS[b]		
	Input	Extract	Pellet	Input	Extract	Pellet
Protein (mg)	3.20	1.68	1.66	3.54	1.72	1.74
(%)	(100)	(52.5)	(51.8)	(100)	(48.6)	(49.1)
Neutral sugar (mg)	0.365	0	0.361	0.327	0.012	0.321
Sialic acid (nmoles)	250	0	243	477	32	472
Phospholipid (μg P)	62.4	0.16	57.1	64.1	0	60.8

[a]One ml of ghosts plus 9 ml 0.1 N NaOH were incubated for 15 min on ice, then cetrifuged. The supernatant fraction was collected and recentrifuged before analysis; the pellet was resuspended and assayed directly.

[b]One ml ghosts and one ml 50 mM LIS in 56 mM Na borate (pH 8.0) were mixed for 15 minutes at room temperature. Four ml of deionized water was then added and the suspensions mixed for another 10 minutes before centrifugation and processing as with NaOH. Values are averages of duplicate or triplicate determinations.

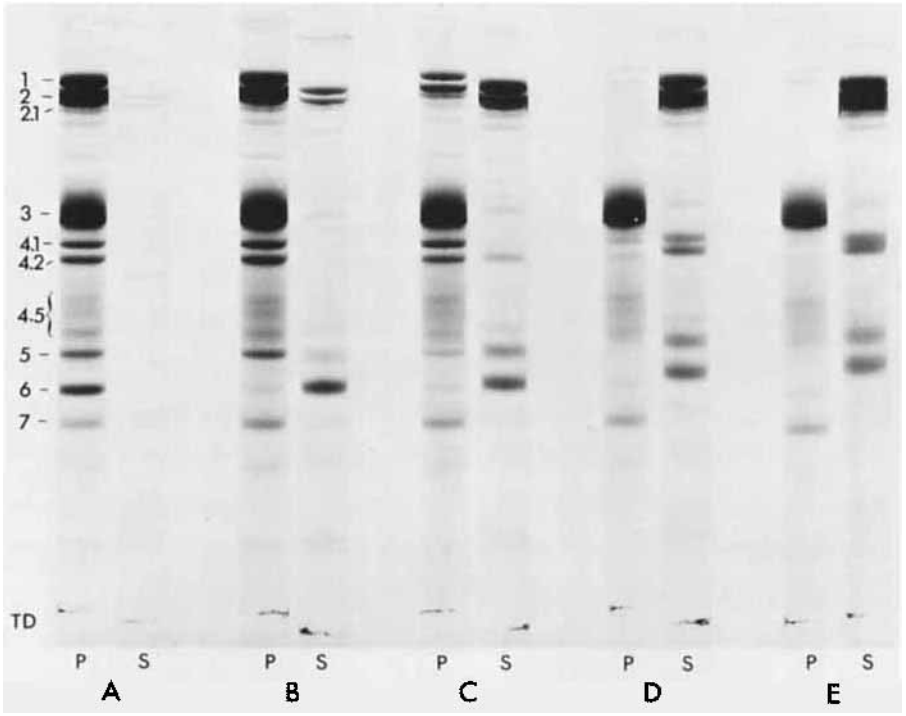

Fig. 2 The release of membrane polypeptides by lithium diiodosalicylate (LIS). One volume of packed ghosts was mixed with seven volumes of LIS in 5P8 and incubated on ice for 30 min. The suspensions were centrifuged and each pellet (P) and supernatant fraction (S) was sampled for electrophoresis. Final LIS concentrations (mM): (A) 0; (B) 5; (C) 10; (D) 20; (E) 40.

relates to the aforementioned finding that heavily glycosylated polypeptides, confined to the membrane residue, do not readily take up the protein stain (6, 22).

The NaOH supernatant fractions were invariably optically clear following low-speed centrifugation. No polypeptide component in the 0.1 N NaOH extract exhibited a sedimentation coefficient greater than 8, even after complete removal of alkali by dialysis against 0.5 mM Na phosphate (pH 8.0).

It can be postulated that if the resistance of certain components to NaOH release reflects their penetration into the membrane core, then the eluted polypeptides might have a more polar amino acid composition while the retained fraction would be relatively enriched in hydrophobic residues. Our preliminary results support this premise (T. L. Steck and H. Köhler, unpublished data). A 0.1 N NaOH eluate contained 40% acidic plus basic amino acids, while the delipidated residue had only 27%. Conversely, the eluate had 27% hydrophobic residues – i.e., val, met, ile, leu, tyr, and phe (2) – compared to 36% in the pellet. The remaining ("neutral") residues amounted to 34% and 37% for the eluted and pelleted fractions, respectively. Similar findings were previously reported by Smith and Verpoorte (24).

Extraction with Lithium 3, 5-Diiodosalicylate (LIS)

LIS is a potent dissociating agent, used by Marchesi and Andrews at 0.3 M to disperse erythrocyte membranes for glycoprotein isolation (25). We used this reagent at low con-

centrations to search for differential polypeptide release. As seen in Fig. 2, polypeptides were eluted by 5 to 20 mM LIS in this order: band **6, 1+2+5, 4.2, 4.1** and **2.1.** Several minor species were dissociated concurrently. At all LIS concentrations up to 60 mM, bands **3, 7** and the poorly resolved zone termed **4.5** were quantitatively retained in the residue. The extracted membranes appeared by phase contrast microscopy to be vesicles, which diminished in size as the LIS level increased. Above 100 mM, no pellet was observed, in agreement with Marchesi and Andrews (25).

The chemical composition of the LIS extract and pellet was essentially the same as that found with 0.1 N NaOH (compare the two sections of Table I). Half of the protein but none of the sugar or phospholipid was eluted by LIS. We have further observed that membrane bound acetylcholinesterase and glyceraldehyde 3-phosphate dehydrogenase activities were completely abolished by LIS; NADH-cytochrome *c* oxidoreductase activity persisted and was partially eluted.

The LIS extracts, like those in NaOH, contained no component with a sedimentation coefficient greater than 8, even after removal of the LIS by dialysis against 0.5 mM Na phosphate (pH 8.0).

Extraction with Acid Anhydrides

Succinic anhydride in 4 M urea has been reported to disperse erythrocyte membrane proteins into the aqueous phase, but a completely monomolecular solution was apparently not achieved (12). Since succinylation replaces free amino groups with carboxylate anions, we conjectured that its action on ghost membranes might resemble that seen with alkali (namely, differential electrostatic dissociation of susceptible proteins).

Preliminary studies with succinic anhydride bore out this premise. However, electrophoretic analysis was unsatisfactory in that the acylated proteins failed to stain well with Coomassie blue (presumably, an electrostatic effect). We therefore repeated the experiment with a related reagent, 2, 3-dimethylmaleic anhydride (DMMA). Since its amide bond to proteins is extremely acid labile (26), this reagent will be released from the polypeptides during routine gel staining in the presence of 10% acetic acid.

Figure 3 summarizes the results. As discussed above, deionized water at pH 8 selectively solubilized bands **1, 2,** and **5.** The addition of 2 mg DMMA/ml ghosts caused the release of band **6** and several trace components, as well as small amounts of bands **2.1, 4.1** and **4.2.** Six to 20 mg of DMMA essentially completed the release of these components. At 60 mg DMMA or 100 mg succinic anhydride/ml packed ghosts, this elution pattern was sustained; however, the quality of the staining profile deteriorated. Bands **3** and **7** plus zone **4.5** were retained in the pellet throughout. The membranes appeared to be small vesicles in the phase contrast microscope.

Extraction with Organic Mercurials

While these studies were in progress, Carter (9) reported that 1 mM pCMBS caused a partial selective release of the same erythrocyte membrane polypeptides eluted by guanidine-HCl (8) and the reagents discussed above. Others (24, 27) had previously noted that organic mercurials liberate a particular fraction of the membrane protein. We have confirmed these observations. Figure 4 (A and B) demonstrates that 5 mM p-chloromercuribenzoate (pCMB) and pCMBS selectively release bands **1, 2, 2.1, 4.1, 4.2, 5, 6,** and certain minor species, leaving components **3, 4.5** and **7** in the residue. The elution is nearly complete at 1 mM pCMBS (Fig. 4C–F).

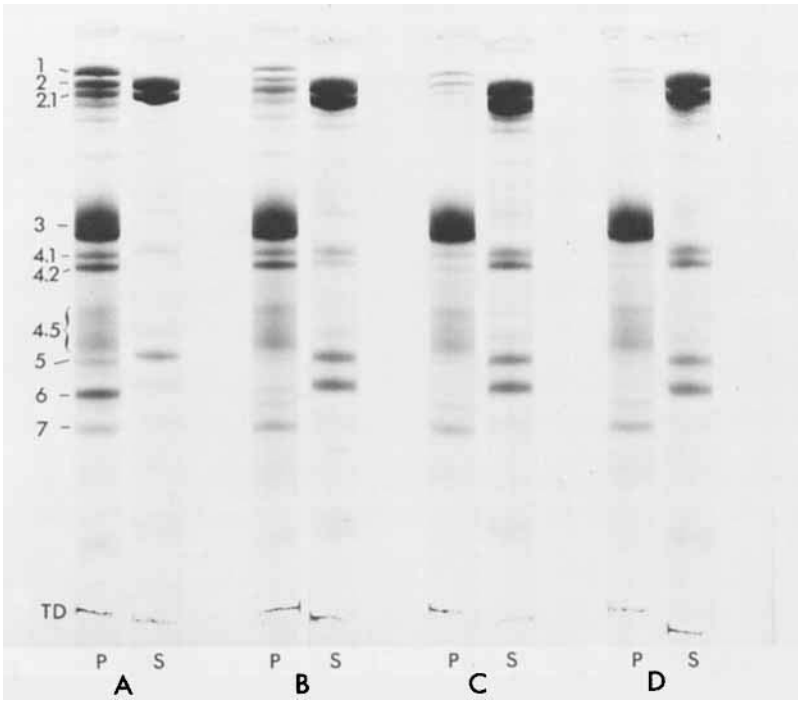

Fig. 3. The release of membrane polypeptides by 2, 3-dimethylmaleic anhydride (DMMA). One ml aliquots of packed ghosts were suspended in seven ml H_2O (at room temperature) and solid DMMA was added graudally while the pH of the mixtures was maintained at 8.0 with 2 N NaOH. After the evolution of acid ceased, the suspensions were centrifuged and each pellet (P) and supernatant fraction (S) was sampled for electrophoresis. DMMA added (mg/ml ghosts): (A) O; (B) 2; (C) 6; (D) 20.

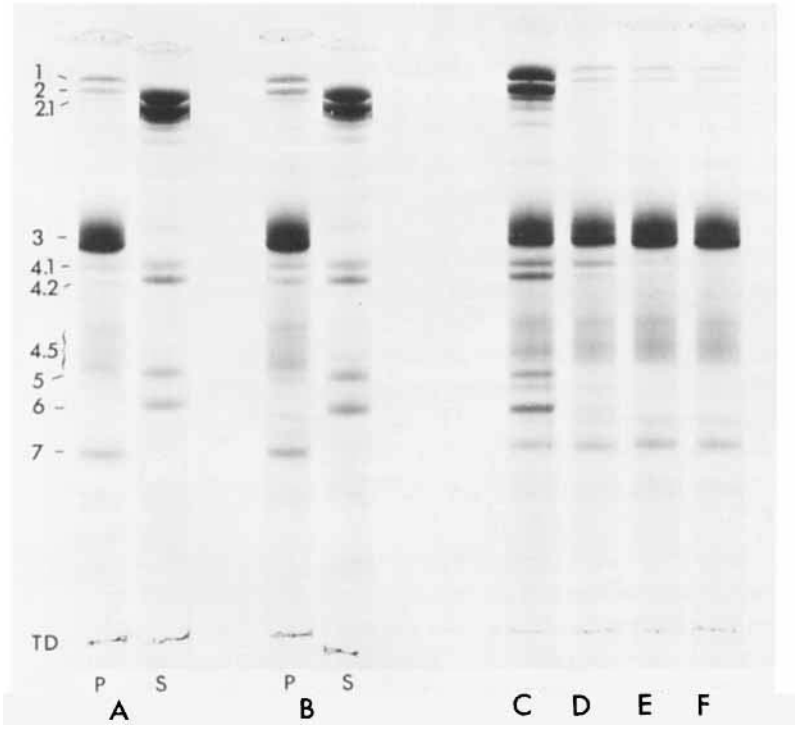

Fig. 4. The release of membrane polypeptides by organic mercurials. Left: Pellet (P) and supernatant fractions (S) derived from ghosts extracted for 30 min. on ice with 7 volumes of 5P8 containing: (A) 5 mM pCMB and (B) 5 mM pCMBS (final concentrations).
Right: Pellets of ghosts extracted for 60 min. on ice with 9 volumes of 5P8 containing pCMBS at (C) 0; (D) 1; (E) 3; and (F) 5 mM.

Consistent with earlier reports (24, 27), we also found that 5mM N-ethylmaleimide (NEM), 5 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 44 mM iodoacetate, and 44 mM iodoacetamide caused no polypeptide elution. The effects of the organic mercurials were blocked by an excess of 2-mercaptoethanol but not by pretreatment of ghosts with 5 mM NEM. It is known that both NEM (28, 29) and iodoacetate (29) alkylate the various major membrane polypeptides at millimolar concentrations. The apparent specificity of action of the organic mercurials is in keeping with other evidence that various thiol reagents react differentially with this membrane (cf 9, 24, 27).

Because of the potency of LIS and the organic mercurials, we explored the possibility that other substituted phenolic compounds could provoke the same elution pattern. However, neither 100 mM Na benzoate, Na salicylate, Li sulfosalicylate nor 20 mM phenol was effective in this regard.

Effect of Protein Perturbants on Glycoproteins

Gels identical to those in Figs. 1–4 were stained with periodic acid-Schiff reagent to determine the fate of the glycoproteins following extraction. Figure 5 summarizes the results. None of the reagents tested (including pCMB, pCMBS, and succinic anhydride, which are not shown) released any of the glycoproteins or PAS-positive lipid. These results are consistent with the fact that all the neutral sugars and sialic acid are retained in the membrane residue (Table I). Furthermore, none of the membrane components specifically labeled by treatment with galactose oxidase plus $^{3}H\text{-}BH_4$ is released by 0.1N NaOH (15; T. L. Steck and G. Dawson, in preparation).

DISCUSSION

We have demonstrated that a wide variety of protein perturbants, namely, NaOH, LIS, acid anhydrides, and organic mercurials, as well as guanidine-HCl (8), releases the same group of polypeptides from isolated human red blood cell membranes. The solubilized species all have sedimentation coefficients of 8 or less. They contribute half of the membrane protein mass, but none of the carbohydrate. The membrane residue retains all the glycoproteins, glycolipids, and phospholipids. The summed polypeptide profile is invariably conserved; i.e., release is not mediated or accompanied by degradation or irreversible aggregation of the proteins. The polypeptides were often released sequentially, offering a facile partial purification of various components.

The membranes vesiculate upon exposure to all perturbants except 0.1 N NaOH. Whether vesiculation signifies merely a transformation in size or a more profound alteration in molecular architecture is not clear. We have previously shown that the vesicles generated during the elution of bands **1, 2,** and **5** in dilute alkaline buffers retain the asymmetrical structure of the parent membrane (15, 23). The stability of ghosts exposed to 0.1 N NaOH was surprising. If, as previously proposed (1, 4), bands **1, 2,** and **5** serve a structural or stabilizing role, then 0.1 N NaOH must somehow compensate for the total elution of these polypeptides from the membrane. The ghost-like configuration is retained even after neutralization with HCl and two water washes. In fact, incubation of the neutralized and water-washed NaOH residue in 50 mM LIS or 0.1 mM EDTA at 37°, treatments which invariably fragment normal ghosts, caused no vesiculation of the NaOH residues. Lysophosphatides generated from membrane phospholipids by alkaline hydrolysis might be a possible source for this unusual stability. In this regard, Wittels (30) has shown

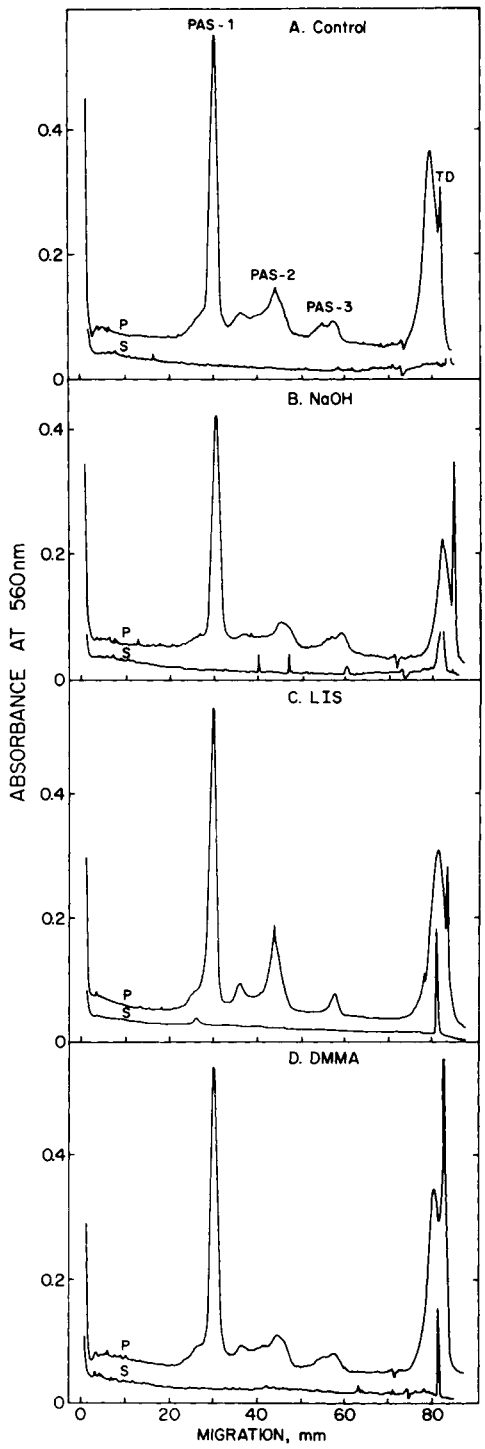

Fig. 5. The release of membrane glycoproteins by various perturbants. One volume of ghosts was diluted with seven volumes of reagent, incubated, centrifuged and processed as indicated in Fig. 1–3, except that the gels were stained with periodic acid-Schiff reagent and scanned at 560 nm, PAS 1–3 are the principal glycoprotein peaks. The unlabeled peak just behind the tracking dye (TD) corresponds to PAS-positive lipids. S=supernatant and P=pellet fractions. (A) 5P8 control; (B) 100 mM NaOH; (C) 40 mM LIS; (D) 6 mg DMMA/ml packed ghosts.

that lysolecithin can reduce erythrocyte fragility associated with primaquine-induced endocytic vesiculation (31).

Out selective elution data are consistent with previous studies using simple aqueous (4–6) or alkaline (24, 32) extraction, 6 M guanidine-HCl (8, 33), and sulfhydryl reagents (9, 24, 27). Acetic acid (34) and urea solutions (32) also resemble somewhat the reagents studied here. [Similarly, Fleischer et al. (35) have shown that acetic acid and urea solutions differentially release from mitochondrial membranes a common set of polypeptides, leaving half of the protein associated with the phospholipids in the membranous residue; the disposition of carbohydrate was not noted.] On the other hand, the selectivity of these interactions stands in strong contrast to previous reports implying rather complete solubilization of red cell membranes with EDTA (13), 6 M guanidine-HCl (14), succinic anhydride (12), and LIS (25). How is it, for example, that 5 mM EDTA was found by Reynolds and Trayer (13) to be more potent in solubilizing the red cell membrane than 6 M guanidine-HCl (8) or 0.1 N NaOH? These apparent discrepancies could reflect differences in the criteria for solubilization or in experimental conditions (e.g., time, temperature, reagent concentration, shearing by vigorous mixing), since the intent of the other studies was total solubilization while the opposite was the case here.

The fact that the disparate reagents all lead to the same elution profile indicates that the specificity of polypeptide release resides to some degree in the membrane, and not merely with the agent. The diversity of these reagents, furthermore, discourages detailed speculation as to how a given protein is bound to the membrane. For example, bands **1**, **2**, and **5** are released by chelation (4, 6, 13), weak alkali (5, 23), dilute acid (34), and organic mercurials (9). Band **6** is selectively released both by raising the ionic strength (6, 7, 29) and by 5 mM LIS (Fig. 2).

It seems reasonable to surmise that bands **1**, **2**, **5**, and **6**, comprising roughly 30% of the protein (6), are the polypeptides most tenuously bound to the membrane, since they can be selectively liberated by simple aqueous solutions lacking denaturants and covalent modifying reagents; furthermore, they are invariably the components most readily released by the perturbants studied here. Their elution behavior agrees with their superficial disposition; bands **1** + **2** + **5** have been identified with a fibrillar "fuzz" lying peripheral to the trilaminar membrane stratum (1, 4), while band **6** corresponds to the reversibly-bound soluble enzyme, glyceraldehyde 3-phosphate dehydrogenase (7). In contrast, bands **2.1**, **4.1**, **4.2**, and others, accounting for approximately 20% of the protein, are only released by protein perturbants. The remaining, glycosylated half of the protein was not solubilized without disruption of the membrane continuum. We discern here a group of membrane proteins which falls between the *peripheral* (or *extrinsic*) and *integral* (or *intrinsic*) categories discussed by Singer (36) and by Capaldi and Vanderkooi (37). This intermediate class are those polypeptides removed only by protein perturbants without dissolution of the membrane core. Of course, generalizations such as these cannot substitute for a detailed understanding of the specific mode of association each protein makes with the membrane.

We have called the reagents under study by a general term, "protein perturbants," since some members of this group clearly act as denaturants while others may not. It has been specifically demonstrated that the ghost polypeptides solubilized by 6 M guanidine-HCl are random coils (38) and that alkaline denaturation of bovine erythrocyte membrane proteins commences near pH 9.5 (39). As in the case of hemoglobin (40) and aspartate transcarbamylase (41), however, organic mercurials may cause the dissociation of polypeptide subunits without gross denaturation.

What limits the potent denaturants from releasing all of the polypeptides; that is, why are 6 M guanidine-HCl and 0.1 N NaOH no more effective than 0.005 M pCMB in liberating the glycoproteins? The most appealing hypothesis requires that none of these treatments disrupts strong hydrophobic lipid-lipid and lipid-protein associations. This premise is not favored by the concept that denaturants such as guanidine-HCl or LIS act by altering the "structure" of the aqueous solvent to better accomodate apolar groups. It is consistent with the argument that these denaturants interact directly with protein constitutents, particularly peptide bonds (cf 42), and would thereby shift the conformation of membrane proteins toward a denatured (dissociable) state if strong apolar associations did not supervene. There is also indirect evidence supporting the hypothesis that the red cell membrane glycoproteins are hydrophobically anchored in the lipid core:

1. At least two of the noneluted polypeptides, bands **3** and PAS-1, are believed to span the thickness of the membrane (15, 43, 44) and would presumably interact with the apolar membrane center in that configuration. The fact that the globular particles seen upon freeze-fracture persist in NaOH-extracted membranes (R. S. Weinstein and T. L. Steck, unpublished data) also suggests that among the noneluted proteins are those which penetrate into or through the lipid stratum.

2. The content of hydrophobic residues is increased in the noneluted over the eluted fraction. Recent reviews have suggested that tightly-bound, integral, or intrinsic membrane proteins are likely to have the greatest quotient of apolar side chains (2, 37). However, the amino acid profile may be misleading. For example, the sialoprotein, PAS-1, is firmly membrane-bound and is believed to span from one membrane surface to the other (15, 43), yet it has a distinctly hydrophilic composition (45, 46). Therefore, elution behavior may better reflect the mode of association of a polypeptide with the membrane than its amino acid composition.

3. Integral components such as **3** (6) and PAS-1 (45) exhibit a strong tendency toward tenacious aggregation, not observed with readily eluted species (cf 2, 36).

4. In an accompanying report (16), we demonstrate that the glycoproteins which resist elution here can be preferentially solubilized by nonionic detergents, leaving a sedimentable lipoprotein residue enriched in those polar polypeptides released by protein perturbation. We surmise that the nonionic detergents disrupt hydrophobic associations between proteins and lipids (without significantly altering protein conformation), and thus effect an elution pattern reciprocal to that of the perturbants.

Finally, it seems more than a coincidence that no glycoprotein is eluted by any perturbant and that the retained components (except band **7**) are all glycoproteins. The Schiff-positive peaks (PAS 1–3) are not the only glycoproteins. The glycosylation of band **3** has been demonstrated by direct chemical analysis (1, 47). Zone **4.5** appears to contain a dispersion of several glycoproteins, as judged by the complex PAS profile and the intense labeling by galactose oxidase plus ^3H–BH_4 in this region of the gel (15; T. L. Steck and G. Dawson, in preparation).

All of the perturbant-released components are localized to the inner (cytoplasmic) membrane surface (cf 15). In contrast, all of the noneluted species (except band **7**) are accessible at the external membrane surface. Components **3** and PAS-1 penetrate the membrane asymmetrically, with their carbohydrate groups oriented to the external surface. Our working model of the disposition of the major proteins in the red blood

cell membrane (15), like that of Juliano and Rothstein (32), identifies the readily dissociated components as the class of nonglycosylated polypeptides at the cytoplasmic surface. The set of glycoproteins comprising the nonelutable polypeptides are exposed at the outer surface but penetrate asymmetrically into and, in at least two cases, through the hydrophobic membrane core. Band **7** is the sole exception; though it is a nonglycosylated, inner surface constituent, it is not eluted by these perturbants.

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