Transfer of Band 3, the Erythrocyte Anion Transporter, between Phospholipid Vesicles and Cells†

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Appendix: Analysis of Chloride Influx

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ABSTRACT: Band 3, the anion transport protein of human erythrocyte membranes, can be transferred from cells to liposomes and from liposomes back to cell membranes, retaining function and native orientation. After incubation with cells, sonicated phosphatidylcholine vesicles bind a transmembrane protein that comigrates with band 3 on sodium dodecyl sulfate–polyacrylamide gels. Like native red cell band 3, the vesicle-bound protein is cleaved by chymotrypsin into 65- and 30-kdalton fragments and is not cleaved by trypsin. The protein can be cross-linked by copper–phenanthroline oxidation either before or after transfer to vesicles; in either case, the vesicle fractions contain high molecular weight material that is dissociated into 95-kdalton species by mercaptoethanol. Band 3–vesicle complexes contain no detectable cell lipid and are specifically permeable to anions. Greater than 99% of their anion uptake can be blocked by the band 3 inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS). Red cells whose band 3 function has been blocked irreversibly by DIDS or eosin maleimide regain part of their anion permeability upon incubation with band 3–vesicle complexes. Under the conditions employed, an average of one copy of functional band 3 is delivered to half of the cells, increasing by 2.3-fold the number of cells containing functional anion transporters. Incubation of pure lipid vesicles or red cell membrane buds with either normal red cells or eosin maleimide inhibited cells has no detectable effect on the cells' anion permeability.

Transfer of intrinsic membrane proteins from red cells to model membranes is a spontaneous process governed by the relative fluidity of the participant membranes (Bouma et al., 1977; Cook et al., 1980). Under the conditions described in the foregoing reports, interaction between cell and vesicle membranes results in transfer of phospholipid to the cells and formation of a heterogeneous population of lipid–protein complexes in the supernatant. Some of these complexes are cell fragments (buds) depleted of cytoskeletal proteins and enriched in acetylcholinesterase (Lutz et al., 1977; Ott et al., 1981; Bouma et al., 1977), while others contain cell membrane protein and lipid derived from the model membranes (this work). These populations differ in density and can be separated by gradient centrifugation or affinity chromatography (K. J. Kim and W. H. Huestis, unpublished results). Their relative abundance depends on experimental parameters such as temperature, model membrane lipid composition, and concentration. Membrane fragments of the first type are produced under a variety of conditions associated with red cell crenation, including ATP depletion and calcium loading (Allan et al., 1976) as well as foreign lipid uptake. The second type of complex is observed only when liposomes are present to act as recipients for the proteins; such species are not generated by monomeric lipid introduced into the cell in the absence of exogenous aggregates (Bierbaum et al., 1977).

At least four major polypeptides transfer readily from human red cells to phosphatidylcholine vesicles (Bouma et al., 1977). Two of these comigrate with bands 4.2 and 7 on sodium dodecyl sulfate–polyacrylamide gels. A third is the acetylcholinesterase (AChE),[1] an intrinsic red cell membrane protein (Ott et al., 1975) that migrates at 81 kdalton on such gels. Up to 90% of this enzyme can be transferred from red cells to exogenous lipid aggregates, remaining fully active with its catalytic site exposed to impermeant substrates.

The fourth major species appearing in the vesicle fraction is band 3, the protein responsible for chloride–bicarbonate exchange in red cells [reviewed by Cabantchik et al. (1978)]. Band 3 is an abundant transmembrane protein that migrates as a diffuse band around 95 kdalton on sodium dodecyl sulfate–polyacrylamide gels. In early studies of protein transfer, a 90-kdalton protein was observed in the vesicle fraction but was not identified as band 3 due to anomalous narrowing in the gel system used (Bouma et al., 1977). Functional assays and enzymatic digestion studies described in this work revealed that this species is in fact the anion exchange protein.

In its native configuration, band 3 has contrasting characteristics at the inner and outer membrane surfaces, indicative of a specific vectorial orientation in the lipid bilayer. Chymotrypsin cleaves the protein into two fragments (30 and 65 kdalton) at a site accessible from the outside but not the inside of the membrane (Cabantchik & Rothstein, 1974). Conversely, trypsin cleaves band 3 from the cytoplasmic face but not the outer face of the membrane, yielding large (48–58 kdalton) and small peptide fragments (Cabantchik & Rothstein, 1974). Band 3 can be cross-linked by sulfhydryl oxidation, a reaction that is most efficient when the cytoplasmic membrane face is directly accessible to the oxidizing agent

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[1] Abbreviations: AChE, acetylcholinesterase; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; eosin MA, eosin maleimide; Con A, concanavalin A; β-ME, β-mercaptoethanol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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(Steck, 1972). Specific anion transport inhibitors such as 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) and eosin maleimide (eosin MA) block anion transport from the outer but not the inner membrane surface (Whitely & Berg, 1974; Nigg & Cherry, 1979).

The studies described here examine the orientation and function of the 95-kdalton protein that transfers to phospholipid vesicles, investigate the effects of protein transfer on vesicle permeability to anions and other solutes, and explore the possibility that proteins can be transferred in the reverse direction, from vesicles to cell membranes. This last possibility was raised by the observation that radioactively labeled protein adheres to cells following incubation with iodinated vesicle–protein complexes (Huestis, 1977). The present work demonstrates further that red cells whose band 3 function has been blocked by an irreversible inhibitor regain part of their anion permeability on incubation with functional band 3–vesicle complexes.

Materials and Methods

Materials

Dimyristoylphosphatidylcholine (DMPC) bovine chymotrypsin, bovine trypsin, and *o*-phenanthroline were products of Sigma Chemical Co. $Na_2{}^{32}PO_4$ and Aquasol were purchased from New England Nuclear, $Na^{36}Cl$ was from ICN, [^{14}C]-dipalmitoylphosphatidylcholine (DPPC) was from Amersham Radiochemicals, DIDS was from Aldrich, eosin MA was from Molecular Probes, and concanavalin A–Sepharose 4B (Con A–Sepharose) and Sephadex G-25 were from Pharmacia. All other chemicals were reagent grade. Human erythrocytes were obtained from adult volunteers, prepared as described (Bouma et al., 1977), and used within 36 h of collection. Unless otherwise stated, experiments were conducted in phosphate-buffered saline containing 138 mM NaCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 5 mM KCl, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.4 ($NaCl/P_i$ buffer).

Methods

Protein Transfer from Cells to Vesicles. Unilamellar DMPC vesicles were prepared by sonication of a suspension of the lipid (24–29 mM in $NaCl/P_i$, as noted) in a bath sonicator (Bouma et al., 1977). In some experiments, the sonication medium contained a radioactive phospholipid marker (0.42 μCi of [^{14}C]DPPC mL^{-1}, 115 mCi mmol^{-1}). Vesicles were incubated with intact red cells (hematocrit 50, unless otherwise noted) or resealed ghosts at 37 °C for 60 min.

Analysis of Vesicle–Protein Complexes. (*a*) *Vesicle Characterization.* Cell–vesicle mixtures (hematocrit 65) were centrifuged at 3000*g* for 5 min to pellet cells, and vesicle supernatants were again centrifuged at 3000*g* for 5 min to remove any contaminating cells. Band 3–vesicle complexes were isolated by applying vesicle supernatants to a DMPC-saturated Con A–Sepharose column (0.9 × 30 cm) and washing the resin with at least three column volumes of a buffer containing 0.2 M NaCl, 10 mM Hepes, 0.7 mM each of $CaCl_2$, $MnCl_2$, and $MgCl_2$, and 5% NaN_3. Band 3–vesicle complexes were eluted with a similar buffer containing 0.1 M NaCl and 0.1 M methyl α-mannoside. The recovered band 3–vesicle fraction typically represented 10% of the total vesicle population. Both adherent and nonadherent vesicle fractions were layered on a sucrose step gradient (10–45% w/v in $NaCl/P_i$) and centrifuged at 27000*g* for 30 min to remove cell fragments or buds. (The adherent vesicle fraction was found to contain no detectable membrane buds; under the conditions employed, these species appear not to adhere to the Con A column.) Vesicle fractions, which banded at 10–15% sucrose, were analyzed for protein [Bradford (1976), using Bio-Rad reagents], phospholipid (Bartlett, 1958), cholesterol (Zlatkis et al., 1953), AChE (Ellman et al., 1961), and [^{14}C]DPPC (by liquid scintillation counting). The amount of band 3 in protein–vesicle complexes was determined from the eosin fluorescence in vesicles after incubation with eosin MA labeled red cells.

(*b*) *Band 3 Orientation and Quaternary Structure.* Intact cells were suspended to a hematocrit of 50 in $NaCl/P_i$ buffer. Chymotrypsin or trypsin was added to yield a concentration of 200 μg mL^{-1}, penicillin G and streptomycin (100 μg mL^{-1}) were added, and the suspension was agitated gently at 22 °C for 14 h. The cells were pelleted and washed 3 times by resuspension in 10 volumes of $NaCl/P_i$ buffer. Protease-digested cells were incubated with an equal volume of DMPC vesicles (24 mM lipid) for 60 min at 37 °C, and then cells were separated from vesicles by centrifugation.

Concurrently, undigested cells were incubated with DMPC vesicles. After separation from cells, half of this vesicle fraction was treated with chymotrypsin or trypsin (200 μg mL^{-1}) for 1 h at 22 °C. The vesicle samples were applied to sucrose density gradients (5–42% w/v in $NaCl/P_i$) and centrifuged at 200000*g* for 3 h at 4 °C. This treatment removed cell fragments (pelleting at 42% sucrose) and enzyme from the vesicles, which banded around 10% sucrose. Vesicle-containing fractions were collected, suspended in 10 volumes of water, and pelleted by centrifugation at 27000*g* for 15 min.

Unsealed ghosts and Triton X-100 (0.5%) solubilized protein–vesicle complexes were also incubated with trypsin to allow the enzyme access to the cytoplasmic face of the membrane. Stroma were prepared from the normal and enzyme-digested cells by lysis and repeated washing with 5 mM Na_2HPO_4, pH 8.0. Stroma and vesicle samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Ames, 1974), and proteins were detected by Coomassie blue or silver staining, as noted.

For Cu(II)–phenanthroline cross-linking, red cell ghosts were prepared as described by Steck & Kant (1974). Unsealed ghosts were incubated for 30 min at 0 °C in 10 volumes of 5 mM Na_2HPO_4, pH 8.0, containing 50 μM $CuSO_4$ and 200 μM *o*-phenanthroline. The membranes were pelleted, washed once, and resealed by suspension in 8 volumes of 5 mM Na_2HPO_4 plus 1 volume of a solution containing 1.4 M KCl, 0.2 M NaCl, 10 mM Tris, and 10 mM Na_2ATP, pH 7.4. After 5 min at 0 °C, the suspension was incubated for 1 h at 37 °C. The resealed ghosts were pelleted, washed once with $NaCl/P_i$ buffer, and incubated with sonicated vesicles as described previously. Normal resealed ghosts were prepared and incubated with sonicated vesicles for 40 min at 37 °C. Vesicles were separated from ghosts and membrane fragments by density gradient centrifugation (5–30% sucrose) as described above. The vesicle fraction from normal ghosts was made 50 μM in $CuSO_4$ and 200 μM in *o*-phenanthroline and then incubated at 0 °C for 30 min. Ghosts and vesicles were subjected to gel electrophoresis under reducing and nonreducing conditions.

(*c*) *Vesicle Anion Permeability.* Anion flux into vesicles was measured by a modification of the technique of Wolosin et al. (1977). After incubation with cells, vesicles (0.5 mL) were added at 25 °C to 0.5 mL of $NaCl/P_i$ or to the same buffer made 2 mM in DIDS. Flux measurements were initiated by pipetting 3 μCi of $Na_3{}^{32}PO_4$ into the rapidly stirred suspension. At specified intervals, 100-μL aliquots were removed and anion flux was quenched by addition to an equal volume of 2 mM DIDS in $NaCl/P_i$ at 0 °C. Aliquots (50 μL)

of these samples were applied to 1.5-mL DEAE-Sephadex A-25 columns at 4 °C. Vesicles were eluted by addition of a precalibrated volume of $NaCl/P_i$ + 2 mM DIDS. The eluted vesicles were collected directly into scintillation vials, dissolved in Aquasol, and analyzed by liquid scintillation counting.

Protein Transfer from Vesicles to Cells. Band 3–vesicle complexes were isolated from whole vesicle fractions as described above, by elution from Con A–Sepharose with methyl α-mannoside. To inhibit vesicle-bound band 3, half of the sample was treated with 0.7 mM eosin MA at 22 °C for 1 h in the dark (Nigg & Cherry, 1979). Unreacted eosin MA was removed by passing the sample through a Sephadex G-25 column. Eosin MA was used as a covalent band 3 inhibitor in this experiment because, unlike DIDS, it is monovalent and does not cross-link and aggregate band 3 enriched vesicles.

Intact red cells were incubated with an equal volume of 0.7 mM eosin MA in $NaCl/P_i$ for 3 h in the dark at 22 °C and isolated by centrifugation and washing in $NaCl/P_i$. Samples of eosin MA inhibited cells were incubated for 45 min at 37 °C with an equal volume of (a) native band 3–vesicle complexes, (b) eosin MA inhibited band 3–vesicle complexes, or (c) $NaCl/P_i$ buffer; phospholipid concentrations in samples a and b were 0.35 mM. Control samples of normal cells were incubated concurrently with either $NaCl/P_i$ or the same buffer containing pure sonicated vesicles at the same lipid concentration as in experimental samples. All cell samples were pelleted, washed 3 times by resuspension in $NaCl/P_i$, and used immediately for chloride flux measurements.

Analogous experiments were performed with red cell membrane fragments (buds) formed by incubation of red cells with calcium (1 mM) and the ionophore A23187 (50 μM; Allan et al., 1976). Half of the isolated buds were treated with eosin MA, as above, and separated from unreacted reagent by gel filtration. Samples of eosin-inhibited red cells were incubated with $NaCl/P_i$ buffer or with buds containing either functional or inhibited band 3, for the same duration and at the same temperature and band 3 concentration used in vesicle incubations. Cells were isolated and used immediately for chloride flux measurements.

Measurement of Chloride Flux into Cells. Red cells (500 μL) were suspended in rapidly stirred $NaCl/P_i$ buffer (4.5 mL) on ice. $Na^{36}Cl$ (1.9 μCi) was added. At specified intervals, 500-μL aliquots of the suspension were pipetted into 0.2 mM DIDS (900 μL) in $NaCl/P_i$. The cells were pelleted and washed by resuspension in 1.0 mL of 0.2 mM DIDS in $NaCl/P_i$. Aliquots (20 μL) of packed cells were lysed in 0.5 mL of water, dissolved in 10 mL of Aquasol, and analyzed by liquid scintillation counting.

Data Analysis. Cell chloride uptake data were analyzed by a nonlinear least-squares fit of the equation

$$cpm_t = cpm_0 + F cpm_\infty (1 - e^{-kt})$$

where cpm_0 and cpm_t represent the amount of radioactive chloride entrapped in cells at times zero and t; cpm_∞ is taken as chloride trapped in normal cells at equilibrium and is assumed to be constant for all flux measurements. F is the fraction of cells equilibrating chloride, and k is the apparent rate constant for chloride influx. DIDS-insensitive background uptake (as shown by the closed symbols in Figures 6 and 7) was subtracted from net uptake for the purpose of these calculations.

Results

Transfer of Band 3 from Red Cells to Vesicles. The polyacrylamide gel in Figure 1 shows proteins released from intact red cells incubated with DMPC vesicles. Lane 1 contains washed stroma, showing the usual array of major protein bands ranging from 30 to 240 kdalton. Lanes 2–6 show protein appearing bound to whole vesicle fractions at 0, 15, 30, 45, and 60 min of incubation with cells at 37 °C. The most prominent transferred component comigrates with band 3, showing the same diffuse distribution around 95 kdalton. Analysis of eosin MA binding indicates that the transferred band 3 typically represents approximately 5% of total cell band 3.

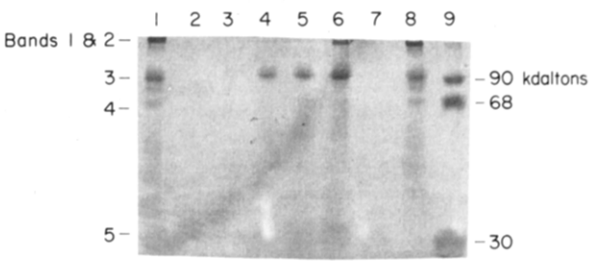

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel (10%) of erythrocyte membranes (lanes 1 and 8) and whole vesicle supernatants after incubation with intact erythrocytes for 0 (lane 2), 15 (lane 3), 30 (lane 4), 45 (lane 5), and 60 (lane 6) min at 37 °C. Prior to electrophoresis, vesicles were separated from unbound protein (lane 7) by passage through Sepharose 4B. Molecular weight standards (lane 9) are phosphorylase *a* (90 kdalton), bovine serum albumin (68 kdalton), and carbonic anhydrase (30 kdalton).

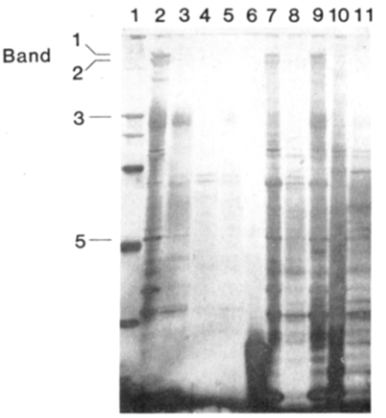

FIGURE 2: Polyacrylamide gel of normal, chymotrypsin-treated, and trypsin-treated band 3 in cells and vesicles: molecular weight standards (lane 1); normal ghost membranes (lane 2); vesicle extract of normal ghosts (lane 3); chymotrypsin-treated vesicle extract (lane 4); trypsin-treated vesicle extract (lane 5); trypsin-treated solubilized vesicle extract (lane 6); chymotrypsin-treated ghosts (lane 7); vesicle extract of chymotrypsin-treated ghosts (lane 8); trypsin-treated ghosts (lane 9); trypsin-treated unsealed ghosts (lane 10); vesicle extract of trypsin-treated intact ghosts (lane 11). All vesicle samples were isolated from cell fragments by ultracentrifugation prior to electrophoresis.

Chymotrypsin treatment of intact red cells or resealed ghosts cleaves band 3 into two fragments of approximately 65 and 30 kdalton (Cabantchik & Rothstein, 1974). Chymotrypsin cleaves the vesicle-bound band 3 similarly, producing the same smaller polypeptides (Figure 2, lane 4). Vesicles incubated with chymotrypsin-digested cells (lane 7) bind the 65-kdalton fragment (lane 8). Trypsin, which does not cleave band 3 in intact red cells (lane 9), produces several proteolytic fragments when applied to unsealed ghosts (lane 10). Trypsin treatment of protein–vesicle complexes has no effect on band 3 (lane 5), while similar treatment of solubilized band 3 complexes results in complete degradation of the protein (lane 6).

Oxidative cross-linking of band 3 does not prevent its transfer to vesicles. The polyacrylamide gel in Figure 3 shows proteins of red cell stroma cross-linked by Cu(II)–phenanthroline, after gel electrophoresis in the absence (lane

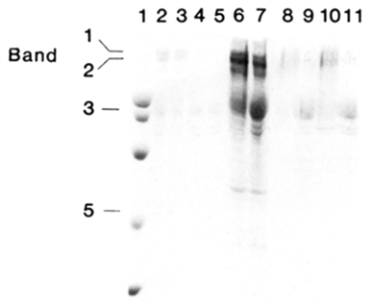

FIGURE 3: Polyacrylamide gel of normal and Cu(II)–phenanthroline cross-linked band 3 in cells and vesicles: standard (lane 1); normal ghosts in the absence (lane 2) and presence (lane 3) of β-mercaptoethanol; vesicle extract of normal ghosts in the absence and presence of β-ME (lanes 4 and 5); Cu(II)–phenanthroline-treated ghosts in the absence and presence of β-ME (lanes 6 and 7); vesicle extract of Cu(II)–phenanthroline-treated ghosts in the absence and presence of β-ME (lanes 8 and 9); Cu(II)–phenanthroline-treated vesicle extract of normal ghosts in the absence and presence of β-ME (lanes 10 and 11). All vesicle samples were isolated from cell fragments by ultracentrifugation prior to electrophoresis.

Table I: Analysis of Vesicle–Protein Complexes

	sonicated vesicles	vesicle complexes not adherent to Con A	vesicle complexes adherent to Con A	red cell membranes[a]
[protein] (mg mL^{-1})	<0.01	0.66	0.15	3.5
[phospholipid] (μmol mL^{-1})	36.4	4.8	1.8	4.3
[^{14}C]DPPC concn (cpm mL^{-1})	1.05×10^6	1.39×10^5	5.0×10^4	
[cholesterol] (μmol mL^{-1})	<0.01	<0.03	<0.01	3.9
protein: phospholipid	<0.001	0.14	0.08	0.8
[^{14}C]DPPC: phospholipid	2.9×10^4	2.9×10^4	2.9×10^4	
cholesterol: phospholipid	<0.001	<0.01	<0.01	0.91

[a] Data taken from Pennell (1974).

6) and presence (lane 7) of β-mercaptoethanol. Consistent with earlier reports (Steck, 1972), most of band 3 is replaced by higher molecular weight material unless the sample is reduced. Vesicles incubated with Cu(II)–phenanthroline-treated ghosts show the same protein pattern; high molecular weight bands are present in the unreduced sample (Figure 3, lane 8), but band 3 reappears when the sample is reduced (lane 9). Thus, band 3 multimers can transfer from Cu(II)-oxidized cells to vesicles.

Band 3 is also cross-linked in band 3–vesicle complexes treated with Cu(II)–phenanthroline (Figure 3, lane 10). The high molecular weight complex produced by this treatment is dissociated into the usual 95-kdalton band by β-ME (lane 11), indicating that a species susceptible to cross-linking transfers to vesicles.

Protein and Lipid Analysis of Band 3–Vesicle Complexes. Sonicated vesicles containing [^{14}C]DPPC as a vesicle lipid marker were incubated with red cells for 60 min at 37 °C. After removal of cells, band 3–vesicle complexes were separated from membrane buds by elution from Con A–Sepharose. Cell fragments were removed from nonadherent vesicles by sucrose density gradient fractionation. Vesicle lipid and protein were analyzed in adherent and nonadherent vesicles and in sonicated vesicles not incubated with cells. The results of these

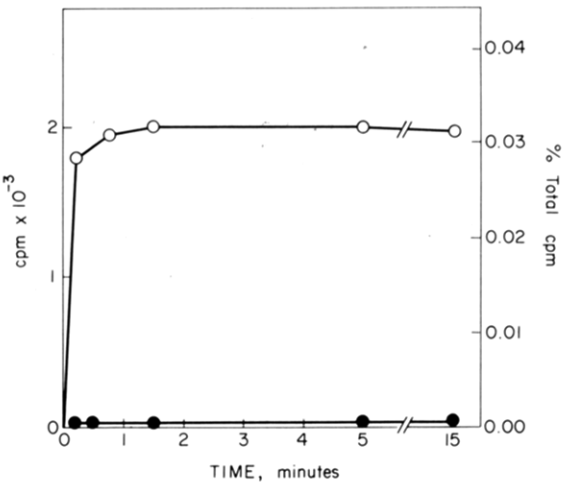

FIGURE 4: [^{32}P]Phosphate flux into band 3–vesicle complexes at 25 °C in the absence (○) and presence (●) of 2 mM DIDS.

analyses are summarized in Table I. The vesicle–protein complexes had similar protein and lipid compositions, differing markedly from the red cell membrane in their lipid:protein ratios and lipid composition. Cholesterol, which comprises approximately 50% of total red cell membrane lipid, could not be detected in the protein–vesicle complexes, and the lipid:protein ratio in vesicle complexes was much higher than in cells. Where vesicle lipid had been labeled with [^{14}C]DPPC, the specific activity of phospholipid in adherent and nonadherent vesicle fractions was indistinguishable from that of vesicles not incubated with cells (Table I).

Anion Permeability of Band 3–Vesicle Complexes. After incubation with erythrocytes, vesicles exhibited DIDS-sensitive anion uptake (Figure 4). Vesicle–protein complexes incubated with $^{32}PO_4^{3-}$, inhibited with DIDS after specified intervals, and separated from excluded phosphate by passage through Sephadex A-25 contained approximately 0.03% of total supernatant anions at equilibrium (2 min at 22 °C). Separation of vesicles from buds by centrifugation revealed that >90% of the entrapped anion was associated with the vesicle fraction. At 13 mM phospholipid, sonicated 250-Å vesicles enclose about 0.5% of the suspending volume. Thus, the equilibrium anion uptake in this experiment corresponds to equilibrium of 6% of the enclosed vesicle volume with the external volume. This anion trapping is not observed in the presence of DIDS (Figure 4) or in vesicles not exposed to erythrocytes.

Transfer of Band 3 from Protein–Vesicle Complexes to Red Cells. Vesicles (170 μg of protein, 0.35 mM phospholipid) were recovered from a Con A–Sepharose column and incubated with red cells whose native anion transport had been inhibited covalently with eosin MA (Figure 5). A second sample of inhibited cells was incubated with protein–vesicle complexes previously inhibited with eosin MA, and a third sample was incubated similarly in NaCl/P_i. Normal cells were incubated under the same conditions with pure phospholipid vesicles or buffer. After these incubations, the chloride permeabilities of the normal cell samples were indistinguishable. Inhibited cells exposed to functional band 3–vesicle complexes showed enhanced chloride uptake relative to inhibited cells incubated with either buffer or inhibited band 3–vesicle complexes (Figure 6). After incubation with the functional complexes, 46 ± 4% of the eosin MA inhibited cells equilibrated their chloride space with external anion, compared with 20 ± 4% of such cells incubated in buffer or with inhibited complexes. The rate constants of chloride uptake were similar for all three inhibited cell samples (0.087 ± 0.001 and 0.086

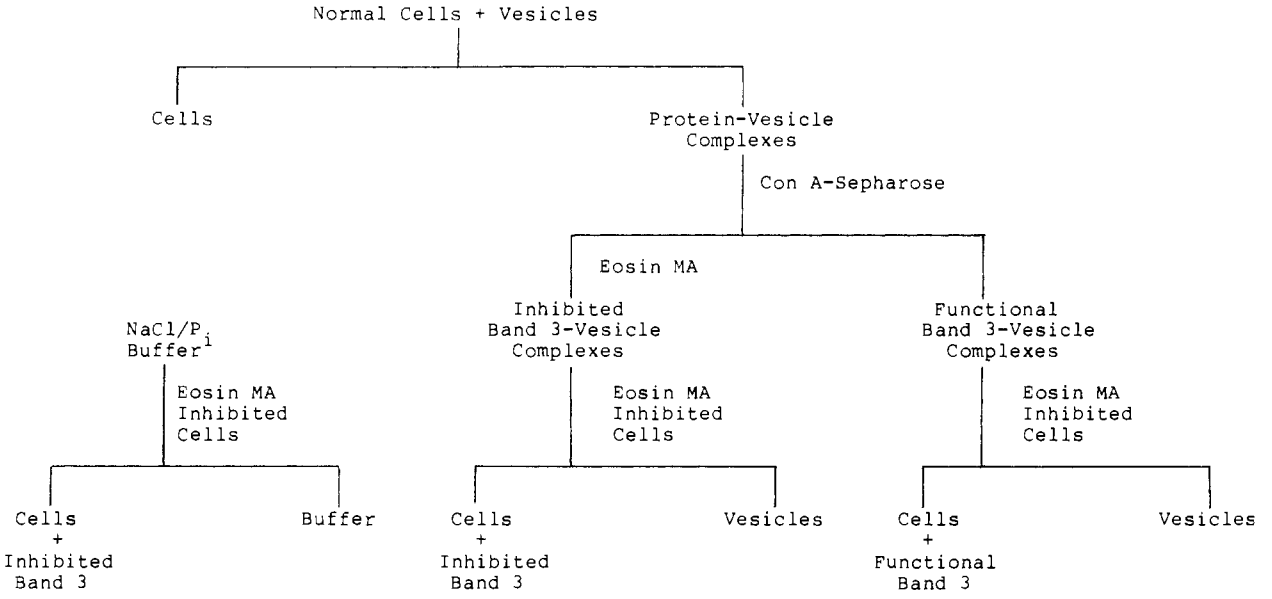

FIGURE 5: Protocol for reverse transfer of functional band 3 to irreversibly inhibited red cells. Eosin MA was used to inhibit band 3 in this experiment because, unlike DIDS, it is monovalent and does not cross-link and aggregate the band 3–vesicle complexes.

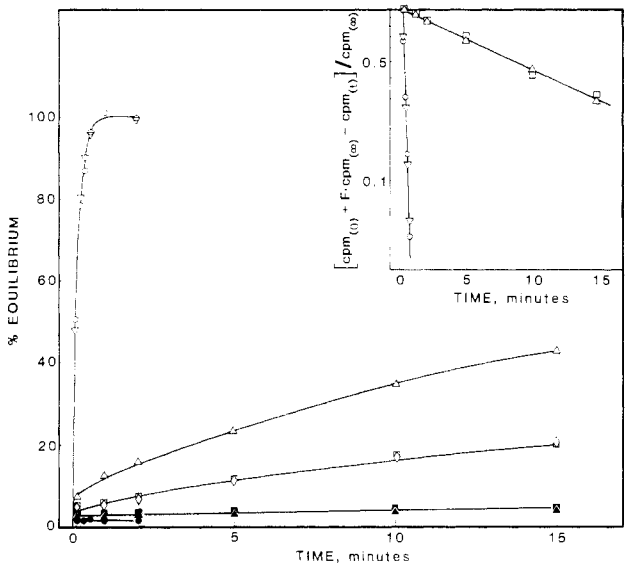

FIGURE 6: Chloride uptake into cells at 0 °C: normal erythrocytes (○); normal erythrocytes after incubation with DMPC vesicles (▽); eosin MA inhibited cells after incubation with functional band 3–vesicle complexes (△), with eosin MA inhibited band 3–vesicle complexes (□), or with NaCl/P_i (◇). Filled symbols show chloride uptake into the same samples in the presence of 0.2 mM DIDS. Inset: Data are fit to the function $cpm_t = cpm_0 + Fcpm_\infty(1 - e^{-kt})$ where the parameters cpm_0, F, and k are obtained from least-squares analysis.

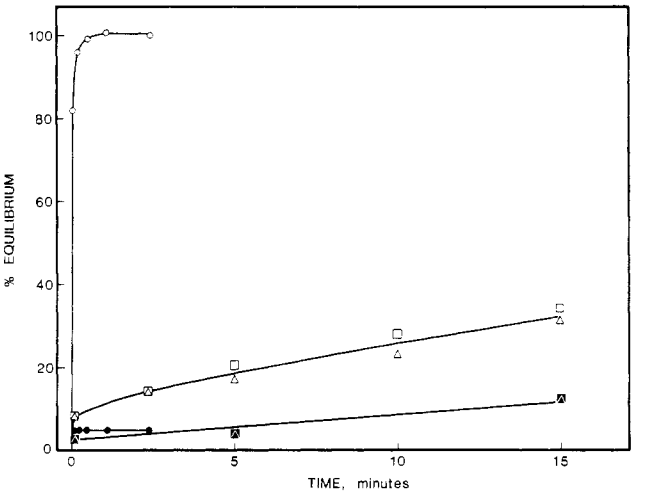

FIGURE 7: Chloride uptake into cells at 0 °C: normal erythrocytes (○); eosin MA inhibited erythrocytes after incubation with normal (△) or eosin-inhibited (□) membrane buds. Cells incubated in buffer show indistinguishable chloride uptake (not shown). Filled symbols show chloride uptake into the same samples in the presence of 0.2 mM DIDS. Background DIDS-suppressible chloride uptake is higher in these samples than in those in Figure 6 due to less nearly complete eosin inhibition.

± 0.004 min^{-1} for the first two, respectively) and equal to 1.7% of the rate constant observed in normal cells (5.1 min^{-1}) (Figure 6, inset). In all cell samples, chloride uptake was inhibited quantitatively (>99%) by 0.2 mM DIDS.

In a separate experiment, eosin-inhibited cells were incubated with red cell membrane fragments produced by ionophore-mediated calcium loading. The concentration of cell fragments was adjusted to yield the same band 3 concentration as employed in band 3–vesicle experiments. Anion permeabilities of eosin MA inhibited cells exposed to "normal" membrane fragments were indistinguishable from permeabilities of such cells incubated with inhibited membrane fragments or buffer (Figure 7). Net anion uptake in this experiment was higher than in the experiment shown in Figure 6 due to less complete initial inhibition of cell band 3.

Discussion

Band 3 Transfers from Cells to Sonicated Vesicles, Retaining Native Orientation and Function. When intact red cells or resealed ghosts are incubated with high concentrations of fluid phospholipid vesicles, a protein comigrating with band 3 appears bound to the vesicle fraction (Figure 1). Several lines of evidence suggest that this protein is the functional anion exchange protein, inserted in the vesicle membrane in the native orientation. Proteolysis of the vesicle–protein complex with chymotrypsin cleaves the 95-kdalton species into 65- and 30-kdalton fragments, indicating that the chymotrypsin-sensitive site remains accessible in vesicles as it was in the cell membrane (Figure 2). As with the native cell membrane, trypsin treatment of intact band 3–vesicle complexes has no effect (Figure 2), while trypsinization of the solubilized complex results in complete degradation of the protein. Thus, the vesicle-bound band 3 is unlikely to be oriented upside down in the bilayer or adsorbed nonspecifically on the vesicle surface,

nor is the vesicle generally permeable to macromolecules.

Band 3–vesicle complexes exhibit enhanced specific anion permeability (Figure 4). At equilibrium, a typical whole vesicle fraction entraps 0.03% of supernatant [^{32}P]phosphate. At the vesicle concentration employed, sonicated vesicles enclose 0.5% of the suspension volume; thus, the observed level of phosphate trapping represents equilibration of 6% of the vesicle contents with the external medium. Vesicle anion uptake is inhibited quantitatively by the specific anion transport inhibitor DIDS. Since DIDS acts exclusively at the external face of the transporter, this complete inhibition is further evidence that the protein is bound to the vesicle membrane in the native orientation. If the protein were oriented randomly, a significant fraction of the vesicles could equilibrate with anions in the presence of DIDS. Anions in these vesicles would not be trapped by the DIDS quench and would begin to exchange as the sample was separated from external [^{32}P]phosphate on the ion-exchange column. The time of passage through this column (35–45 s) is insufficient to permit equilibrium to be reached at 4 °C, so some residual DIDS-insensitive phosphate trapping would be observed. No such trapping is found, so the DIDS binding sites of all functional transporters must be accessible to the suspending medium.

Band 3 cross-linked by Cu(II)–phenanthroline oxidation can be transferred to phospholipid vesicles (Figure 3), and the protein can be cross-linked in the vesicle complex. This indicates that a multimeric (possibly dimeric) form of band 3 is transferred. The vesicle-bound species is cross-linked essentially quantitatively by Cu(II)–phenanthroline regardless of whether the reagent was entrapped in the vesicles during sonication (not shown) or added to the vesicle fraction after incubation with cells. This probably reflects on the membrane permeability of the reagent rather than on the orientation of the protein; indeed, extensive heme oxidation was observed when intact red cells were treated with Cu(II)–phenanthroline.

Lipid and Protein Analysis of Band 3–Vesicle Complexes. After incubation with red cells, [^{14}C]DPPC-labeled band 3–vesicle complexes were separated from membrane fragments and protein-free vesicles by elution from Con A–Sepharose. Relative to the cell membrane, these species were enriched in phospholipid and deficient in cholesterol and protein (Table I). Their phospholipid specific activity was indistinguishable from that of sonicated vesicles not exposed to cells. Under the conditions of time and temperature employed, DPPC does not exchange out of vesicles to a detectable extent (Martin & MacDonald, 1976); hence, band 3 appears bound to a lipid complex derived from the sonicated vesicles.

This result could be obtained if the vesicle sample were contaminated with membrane buds or products of fusion between buds and vesicles. According to Palek and co-workers (Palek et al., 1977), spectrin-free cell fragments exhibit the same integral protein:phospholipid ratio as whole cell membranes. If such species are present in the band 3–vesicle fraction, other cell components (e.g., cholesterol) should also be present in proportion to the observed protein content. Cholesterol assays of protein–vesicle complexes isolated from Con A columns show less than 0.01 μmol of cholesterol mL^{-1}. Data in Table I indicate that if all of the protein in the sample were associated with membrane buds or bud–vesicle fusion products, approximately 10% of their phospholipid should also derive from cell fragments. The cholesterol:phospholipid ratio of red cell membranes is approximately 0.91 (Pennell, 1974), so such a quantity of cell phospholipid should be accompanied by 0.16 μmol of cholesterol mL^{-1}. This value is well within the detection limits of the cholesterol assay, leading to the conclusion that contamination of the Con A adherent fraction with membrane fragments can account for no more than 6% of the measured protein. The composition and properties of the isolated band 3–vesicle complexes suggest that they arise from a transient association between the cell membrane and sonicated vesicles, during which protein is exchanged but the structural integrity of each species is retained.

Transfer of Band 3 from Vesicles to Cells. Red cells whose anion transport function has been blocked covalently with eosin MA recover a small fraction of their DIDS-sensitive anion permeability when incubated with band 3–vesicle complexes (Figures 5 and 6). After incubation with functional vesicle complexes, typically 46 ± 4% of the inhibited cells equilibrate chloride with the medium. Inhibited cells incubated with either buffer or inhibited band 3–vesicle complexes eventually equilibrate 20 ± 4% of their chloride volume with the medium, a "background" flux that varies depending on the extent of preinhibition with eosin MA (compare, for example, Figure 7). The observed ion uptake is not due to vesicles adhering to the cells; their volume could accommodate no more than 0.02% of the supernatant chloride at equilibrium, 2 orders of magnitude less than the observed uptake. The increase in the fraction of cells equilibrating chloride is consistent with delivery of functional band 3 into the membrane of the inhibited cells. In all cases, chloride flux is blocked almost quantitatively by added DIDS, indicating that it is mediated by band 3 that retains native orientation in the membrane.

The regenerated ion flux might arise from an alteration in membrane structure that unmasks buried transporters or reverses eosin MA inhibition. Neither process is likely, since inhibited band 3–vesicle complexes or pure lipid vesicles do not restore permeability. [At the phospholipid concentration employed, pure sonicated vesicles have no effect on normal anion transport (Figure 6).] Further, the restored function is not derived from undetectably low levels of contaminating membrane fragments. The anion permeability of eosin MA inhibited cells is not affected by incubation with red cell membrane buds formed by calcium loading, when present at protein concentrations comparable to those of band 3–vesicle complexes (Figure 7). Perhaps surface glycoproteins or lipids form a steric or electrostatic barrier to close approximation between natural membranes.

While the magnitude of the regenerated chloride permeability in these cells is small relative to normal function, the number of reconstituted proteins it represents is not insignificant. Statistical analysis of rate data for eosin MA inhibited cells before and after treatment with band 3–vesicle complexes reveals an increase in the average band 3 content from 0.2 to 0.6 copy per cell (see the Appendix and Figure 8). The conditions employed to achieve this level of reconstitution may not have been optimal. Prior work (Cook et al., 1980) showed that cell to vesicle protein transfer is rapid and extensive when the recipient membrane is more fluid than the donor. If the same were true of vesicle to cell transfer, manipulation of vesicle composition could produce more successful reverse transfer. Ongoing studies are directed toward devising appropriate conditions.

While membrane fragment to cell transfer of functional epidermal growth factor receptor has been reported in one system (Bishayee et al., 1982), the generality and biological significance of protein transfer are by no means clear. In particular, the roles of the cell cytoskeleton, endocytosis, and membrane turnover in regulating possible effects of such events have yet to be explored. In some circumstances, however, protein transfer may provide a uniquely noninvasive way to

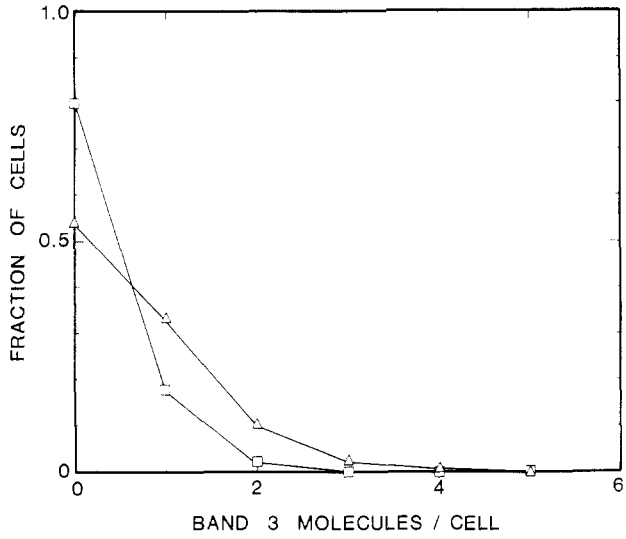

FIGURE 8: Poisson distributions of functional band 3 molecules in eosin MA inhibited cells before (□, μ = 0.22 molecule/cell) and after (△, μ = 0.62 molecule/cell) incubation with band 3–vesicle complexes.

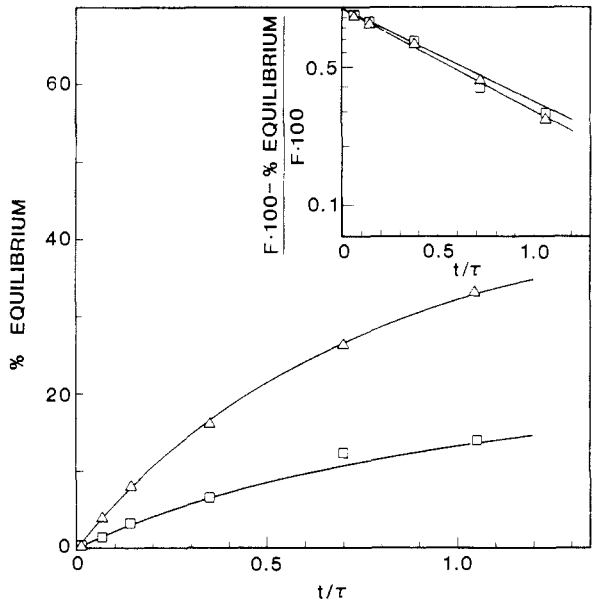

FIGURE 9: Theoretical influx curves constructed from the predicted distributions of functional band 3 molecules shown in Figure 8. The lower curve (□) shows influx into eosin MA inhibited cells (eq A4); the upper curve (△) shows influx into such cells after treatment with band 3–vesicle complexes. The experimental points are taken from Figure 6 after subtracting the t = 0 intercepts and are compared to the theoretical curves by assuming τ = 14.3 s. The inset shows the same theoretical curves and data as a semilog plot. The upper line in the inset corresponds to the lower curve in the main figure.

alter the properties of cell membranes.

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Registry No. Dimyristoylphosphatidylcholine, 13699-48-4; phosphate, 14265-44-2; chloride, 16887-00-6.

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Appendix: Analysis of Chloride Influx

We have presented data showing that eosin MA inhibited cells are made more permeable to chloride by incubation with band 3 containing vesicles. We can now use probability theory to estimate the mean number of functional band 3 molecules per cell and how these molecules are distributed, before and after vesicle treatment.

First, we assume that eosin MA inhibits band 3 molecules in a random fashion and that vesicles deliver functional band 3 molecules one at a time to a random sample of cells. It follows that the fraction of cells containing x functional band 3 molecules is approximated by the Poisson distribution (Bevington, 1969):

$$P(x,\mu) = \mu^x e^{-\mu}/x! \qquad \text{(A1)}$$

where μ is the mean number of functional band 3 molecules per cell.

Prior to incubation with band 3–vesicle complexes, 80 ± 4% (SE) of the eosin MA treated cells showed no anion perme-

ability and presumably contained no functional band 3 molecules. Hence

$$P(0,\mu) = 0.80 \pm 0.04$$

$$\frac{\mu^0}{0!}e^{-\mu} = 0.80 \pm 0.04$$

$$\mu = 0.22 \pm 0.05$$

After vesicle treatment, only 54 ± 4% of the cells remained impermeable to anions, corresponding to an average of 0.62 ± 0.07 functional band 3 molecule per cell. Hence, vesicle treatment produced approximately a 3-fold increase in the mean number of band 3 molecules per cell.

The validity of this model can be tested by using the Poisson distributions corresponding to $\mu = 0.22$ and 0.62 (Figure 8) to reconstruct influx curves analogous to those in Figure 6. Assuming that influx is first order in $[Cl^-]$

$$\% \text{ equilibrium}(t) = 100[1 - \sum_{x=0}^{\sim 10^6} P(x,\mu)e^{-k(x)t}] \quad \text{(A2)}$$

where $k(x)$ is the apparent rate constant for a fraction of cells containing x band 3 molecules per cell. For small x, $k(x)$ should be a linear function of x (eq A3):

$$k(x) = kx = x/\tau \quad \text{(A3)}$$

Hence, for eosin MA inhibited cells

$$\% \text{ equilibrium}(t) \cong 100[1 - 0.800 - 0.178e^{-t/\tau} - 0.020e^{-2t/\tau} - 0.001e^{-3t/\tau} - 0.0001e^{-4t/\tau}] \quad \text{(A4)}$$

Similarly, for cells treated with band 3–vesicle complexes

$$\% \text{ equilibrium}(t) \cong 100[1 - 0.540 - 0.330e^{-t/\tau} - 0.103e^{-2t/\tau} - 0.021e^{-3t/\tau} - 0.003e^{-4t/\tau}] \quad \text{(A5)}$$

These curves, plotted in Figure 9, agree well with the experimental data from Figure 6. It is noteworthy that although these curves explicitly contain four exponential components with different characteristic times, they are indistinguishable from single-component exponentials for the time scales plotted (inset, Figure 9). The ratio of the apparent rate constants before and after vesicle treatment, taken from Figure 9, is 1.20 ± 0.30 (where the standard error is derived from the uncertanties in μ), in agreement with the experimental value of 1.01.

Thermotropic Behavior of Mixed Phosphatidylcholine–Phosphatidylglycerol Vesicles Reconstituted with the Matrix Protein of Vesicular Stomatitis Virus†

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ABSTRACT: The peripheral matrix (M) protein of vesicular stomatitis virus reconstituted with fused unilamellar vesicles containing equimolar amounts of dipalmitoylphosphatidylcholine and dipalmitoylphosphatidylglycerol has been studied by high-sensitivity differential scanning calorimetry and steady-state fluorescence spectroscopy. The association of the basic (p$I \simeq 9.1$) matrix protein with the mixed neutral/acidic phospholipid bilayer induced a dramatic upward shift in the phospholipid phase transition temperature even at M protein/phospholipid molar ratios as low as 1/12000. Despite the large effect of the matrix protein on the phase transition temperature and the shape of the heat capacity function, the enthalpy change associated with the phospholipid gel to liquid-crystalline transition remained constant even at saturating protein concentrations. Steady-state fluorescence depolarization measurements indicated that association of the M protein with the phospholipid bilayer increased the apparent order of the bilayer both below and above the phospholipid phase transition temperature; this effect may be responsible for the observed changes in thermotropic behavior. At high protein concentrations, the matrix protein induced lipid phase separation, probably due to its tight association with the acidic phospholipid component of the membrane.

Vesicular stomatitis virus (VSV)[1] is a negative-stranded RNA virus which is composed of a transcriptase-containing nucleoapsid core surrounded by a lipoprotein envelope. The virion obtains the lipid component of the envelope as it buds from the host cell plasma membrane during viral maturation (Wagner, 1975). Two viral-coded proteins are intimately associated with the envelope: (i) a transmembrane glycoprotein (G) ($M_r \sim 69000$), which comprises the spikes that protrude from the external surface of the membrane (Schloemer & Wagner, 1975), and (ii) a nonglycosylated peripheral matrix protein (M) ($M_r \sim 29000$), which is quite basic (p$I \sim 9.1$; Carroll & Wagner, 1979) and appears to line the internal surface of the viral membrane (Zakowski & Wagner, 1980). Both viral membrane proteins have been purified and reconstituted with phospholipid vesicles of defined composition (Petri & Wagner, 1979; Zakowski et al., 1981).

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[1] Abbreviations: VSV, vesicular stomatitis virus; M, VSV matrix protein; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; SUV, small unilamellar vesicles; FUV, fused unilamellar vesicles; MLV, multilamellar vesicle(s); T_m, lipid phase transition temperature; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; p*I*, isoelectric pH; DPH, 1,6-diphenyl-1,3,5-hexatriene; SDS, sodium dodecyl sulfate; ESR, electron spin resonance.

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