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Analysis of Human Erythrocyte Membrane Vesicles Produced by Shearing

S. L. Schrier and I. Junga

Shearing of ghosts in a French pressure cell produces three classes of microvesicles that differ from endocytic vacuoles, exocytic vacuoles, and inside-out vesicles. It was thought that an analysis of these vesicles might provide some clues about the assembly of proteins within the human erythrocyte membrane. The microvesicles were separated into three visible bands, labeled top, middle, and bottom, and assayed for activity of Mg^{++}-ATPase, Na^+,K^+-ATPase, acetylcholinesterase, glyceraldehyde-phosphate dehydrogenase, and NADH oxidoreductase. Their proteins were also characterized by polyacrylamide gel electrophoresis with both Coomassie blue staining, to assess total protein content and distribution, and PAS-staining, to characterize sialoglycopeptides. In order to minimize problems inherent in ghost preparation, Dodge or hypotonic ghosts and glycol or isotonic ghosts were used in all studies. Middle membrane vesicles most resembled intact ghosts. Top vesicles had reduced levels of NADH oxidoreductase and more PAS-2 at the expense of PAS-1. The bottom vesicle class was very much enriched with PAS-1 at the expense of PAS-2, and PAS-3 was completely absent. In addition bottom vesicles had highest NADH oxidoreductase activity but lowest activity of all the other enzymes measured. These vesicle classes could not have been produced by tangential shearing through the membrane, nor could radial shearing through a membrane in which all proteins were free to move laterally have accounted for the three discrete vesicle classes or for their different patterns of enzymes and proteins. The analysis of the microvesicles produced by shearing is most consistent with radial shearing through membranes where there may be fixed domains superimposed on the basic fluid-mosaic structure.

Key words: human erythrocyte membranes, membrane microvesicles, sialoglycopeptides, protein content of membranes, enzymic content of membranes, acetylcholinesterase, Mg^{++}-ATPase, Na^+,K^+-ATPase, NADH oxidoreductase, GAPD of membranes

The fluid-mosaic model has provided the operational basis for much of the recent work on erythrocyte membranes [1]. It proposes that proteins are not held in fixed positions but rather are imbedded in the lipid of the membrane, where they have considerable freedom to move and rearrange themselves. However, some recent studies have suggested that for brief periods some of the membrane proteins behave as though they were relatively

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fixed in position [2], thereby creating domains in the membrane. For example, exocytic vacuoles produced in intact erythrocytes [3] and endocytic vacuoles generated in white ghosts [4] were found to be spectrin deplete and probably arose from spectrin-depleted domains [5]. Nuclear extrusion in mammalian normoblasts occurs through a zone that has been selectively depleted of its spectrin but not its actin [6].

In order to explore the possibility that relatively fixed relationships between proteins might exist in human erythrocyte membranes, we returned to a prior study in which we hac shown that when human erythrocyte ghosts were disrupted by shearing them in a French pressure cell, microvesicles were created [7, 8]. These vesicles could be separated by centrifugation through a sucrose density gradient into three grossly visible classes that contained different patterns of ATPase activities and somewhat different lipid:protein ratios [8]. On the basis of the fluid-mosaic membrane model, one should have predicted that all vesicles might have had the same lipid:protein ratio and activities of membrane enzymes expressed per milligram of membrane protein would have been the same. Therefore, experiments were designed where normal human erythrocyte membranes were disrupted in the French pressure cell and the resulting vesicle classes were separated and analyzed for their protein content as determined from Coomassie blue and PAS staining of SDS-solubilized membranes subjected to polyacrylamide gel electrophoresis (SDS-PAGE). In addition the activities of five membrane enzymes were measured: Mg^{++}-ATPase and Na^+,K^+-ATPase were taken to indicate the degree of preservation of complex interactions within the membrane vesicles [9, 10]; acetylcholinesterase (AcCh) is a recognized marker of the external membrane surface [11]; NADH oxidoreductase (NADH Ox-Red) is known to be a cytosol facing enzyme [11, 12]; glyceraldehyde-phosphate dehydrogenase (GAPD) is also a cytosol facing enzyme that has the advantage of having a defined membrane-binding site known to be a specific segment of band 3, the major integral membrane protein [12, 13].

Analysis of the vesicle classes produced by pressure shearing indicated that they were each distinct from either endocytic or exocytic vacuoles, or from inside-out vesicles, all of which are depleted of spectrin [3–5]. The associations of peripheral and integral proteins and enzyme activities seen in the three vesicle classes are not simplistically explained by assuming that the different classes are either inside-out vesicles, or right-side-out vesicles, or that any vesicle class represents enrichment of either the outer or inner membrane surfaces. Rather, the patterns seen suggest that there may be some regular associations of proteins within the fluid-mosaic that constitues much of the red cell membrane.

MATERIALS AND METHODS

Preparation of Human Erythrocyte Ghosts

Ghosts prepared by hypotonic means are known to differ from ghosts prepared under isotonic conditions [12]. Therefore all experiments were performed using both hypotonic and isotonic ghosts.

Volunteers, suitably informed according to procedures established and cleared by the Stanford Human Experimentation Committee, donated venous blood, which was drawn into heparinized tubes. The erythrocytes were immediately washed three times in unbuffered 0.154 M NaCl, during each of which washings plasma and buffy coat were removed as completely as possible.

Preparation of Hypotonic Ghosts [14]

The Dodge method was used maintaining a ratio of 40 volumes of hypotonic buffer to one volume of packed RBC. The first two washes were done with 5 mM phosphate buffer pH 8.0, 4°C, and the third was carried out in 7.5 mM imidazole glycylglycine buffer

pH 8.0 at 4°C. The ghosts were then washed for a fourth and final time in a solution chilled to 4°C consisting of 30% sucrose, 20 mM choline chloride, and 5 mM imidazole glycylglycine buffer, pH 7.4. The resulting packed white ghost preparation contained 7–8.5 mg of protein per milliliter as measured by the Lowry method.

Preparation of Isotonic Ghosts [15]

The glycol technique was used exactly as described. After the completion of the lytic steps, the ghosts were washed for a final time in a solution chilled to 4°C consisting of 30% sucrose, 20 mM choline chloride, and 5 mM imidazole glycylglycine buffer, pH 7.4. The resulting ghosts had a pearl-like cast indicating the presence of some residual hemoglobin, and the suspension contained 7–8.5 mg of protein/ml.

Shearing of Ghosts

Ghosts were sheared in a prechilled 40 ml Aminco French pressure cell at a pressure of 500–600 pounds per square inch [8].

Separation of Microvesicle Classes

One ml of sheared membrane suspension was layered on 11 ml of a 30–42% continuous sucrose density gradient, which was formed over a 0.6 ml 60% sucrose cushion. All sucrose solutions were w/v and were dissolved in 20 mM choline chloride and 5 mM imidazole glycylglycine buffer, pH 7.4. By refractometry, the gradient was 26–36.5% sucrose with densities of 1.3742–1.3930, respectively. The gradients were centrifuged in an SW 41 rotor at 41,000 rpm (approximately 286,000g) at 4°C for three hours. Three distinct visible bands were seen in gradients prepared from both Dodge and glycol ghost vesicles. They were labeled top, middle, and bottom according to their position in the centrifuge tube and were harvested with a Pasteur pipette.

In all experiments, protein solubilized by shearing appeared in a clear zone above the top vesicle band. The recovery and distribution of protein in the gradient are shown in Table I. In a prior study [7] the size of the vesicles in each of the bands was examined by transmission electron microscopy and found not to differ appreciably. Each of the three vesicle bands was recentrifuged in an identical gradient to determine if they ran "true." For Dodge ghosts, all of the top band ran true; 84% of the middle band ran true, and 16% appeared as a faint top band; 90% of the bottom band ran true, and approximately 10% appeared as a sharp middle band. For glycol ghosts, all of the top and middle bands ran true, while 72% of the bottom band ran true and the remainder appeared as a distinct middle band. Since there are no unambiguous markers of the vesicle bands, it is not possible to be sure about the extent of cross-contamination. The vesicle bands do not contain released

TABLE I. Recoveries of Protein From Gradients

	Dodge ghosts		Glycol ghosts	
	Protein	Sucrose density	Protein	Sucrose density
Applied to gradients	8.0 mg		8.1 mg	
Recovered from gradients				
Soluble clear zone	1.04 (13)[a]	1.3747	1.87 (23)	1.3747
Top vesicles	1.83 (23)	1.3754	2.36 (29)	1.3756
Middle vesicles	3.25 (41)	1.3775	2.64 (33)	1.3775
Bottom vesicles	1.16 (15)	1.3800	1.52 (19)	1.3815
Total	7.28 (91)		8.39 (104)	

[a]Number in parentheses indicates % of material added.

soluble proteins which are held at the clear zone at the very top of the gradient. Glycol ghosts retain some hemoglobin which is stripped off during the shearing procedure, and this hemoglobin accounts for the increased amounts of solubilized protein seen with sheared glycol ghosts (Table I). When 0.61 mg of Dodge vesicles from the top band were centrifuged at 100,000g for 60 minutes in phosphate-buffered saline, 0.67 mg of protein was recovered in the vesicle pellet. When 1.31 mg of middle band Dodge vesicles were centrifuged, 1.16 mg were recovered in the pellet. Therefore the vesicle classes were not washed after removal from the gradients because there appeared to be only minimal contamination with soluble proteins and because preliminary experiments had shown that washed pelleted vesicles proved to be very difficult to resuspend smoothly and thus gave erratic results in the enzyme assays.

Before assays of membrane-associated enzymes could be carried out, it was important to determine if the vesicles in the three separated classes were inside-out or right-side-out and whether they were sealed or leaky. Prior studies [8] comparing sialic acid released by either neuraminidase or H_2SO_4 yielded essentially similar results, indicating that the vesicles were either all right-side-out or leaky to a relatively large molecule like neuraminidase. In preliminary studies each vesicle class was shown to contain both externally and internally oriented enzymes, and the values were not increased by saponin addition. Therefore, it was deduced that the vesicles in all classes were leaky to the enzyme substrates used.

Analysis of Vesicle Classes

Recognizing that minor variations in ghost and gel preparation can produce apparent differences in protein distribution, rigorous attempts were made to handle samples in a comparable manner [16]. Each of the fractions studied (intact ghosts, sheared ghosts, and the three vesicle classes) were stored, solubilized, electrophoresed, stained, and scanned in parallel. The membrane proteins were solubilized by boiling for 1.5 min in 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol and then subjected to PAGE using the Laemmli buffer system [17]. The lower gel was 9 cm long and 8% polyacrylamide. The upper stacking gel was 1 cm long and 3% polyacrylamide. Electrophoresis was carried out using 0.6 mamp per tube gel. The proteins were stained with Coomassie blue and labeled according to the nomenclature of Fairbanks [18]. Heavily loaded (50–100 μg) tube gels were also stained with the periodic acid-Schiff (PAS) stain to demonstrate the glycoproteins that were labeled 1, 4, 2, 3 in order from the top of the gel [2, 19]. Relative proportions of the different protein classes were established by densitometer scan of both the Coomassie blue and PAS stained gels in the Gilford densitometer model 2520J, and the identified bands were quantitated by cutting out and weighing the area of the designated bands in the densitometer tracing. Substantial differences can be produced according to the pattern of cutting used. Because background staining varied between experiments and would have a greater impact on diffuse protein bands (eg, band 3) than on sharper bands (eg, bands 1 and 2), a straightedge was used to connect the top of the background staining, and the area was cut out and discarded.

Enzyme Assays

Mg^{++}-ATPase and Na^+,K^+-ATPase were assayed by measuring the release of ^{32}Pi from γ-labeled $AT^{32}P$ in the isobutanol benzene method as previously described [7, 8]. Radioisotopic activity of $AT^{32}P$ was adjusted to levels of 5,000–15,000 cpm/nanomole. Acetylcholinesterase was measured by the acetylthiocholine method [20]. NADH-oxidoreductase was measured by the cytochrome technique in the presence of saponin [21]. GAPD was measured in the linear "backward" assay [22].

RESULTS

Protein Analysis of the Ghosts and the Vesicle Classes

Six experiments on six different normal subjects were analyzed using hypotonic (Dodge) ghosts and isotonic (glycol) ghosts. For the Dodge ghosts seven proteins were identified on the Coomassie blue stained gels [23]: spectrin, bands 1 and 2; bands 2.1 and 2.2, spectrin binding proteins; band 3, the major integral membrane protein; bands 4.1 and 4.2, cytoskeletal proteins that may have spectrin binding functions; band 4.5, which may contain catalase and the glucose-transport protein; band 5, actin; band 6, GAPD. Glycol ghosts retain no band 6 and very little GAPD activity. The relative proportion of these proteins in intact and sheared Dodge ghosts and microvesicles separated from Dodge ghosts is shown in Table II, and for glycol ghosts in Table III. With both Dodge and glycol ghosts, the bottom microvesicle class was relatively enriched in spectrin, bands 1 and 2. Glycol ghost vesicles from the bottom of the gradient had somewhat less band 3 protein than vesicles obtained from the top of the gradient. No other changes reached the level of significance.

Sialoglycopeptide Characterization of the Vesicle Classes

Dodge and glycol ghosts and microvesicles were also analyzed for their glycoprotein content (with PAS). Since it is known that minor variations in dissolving, boiling, and handling the protein can result in interconversions of some of these bands [23], all solubilizations, PAGE, and staining were done as batch procedures to assure parallel treatment of all fractions. The relative proportions of glycoproteins in ghosts and microvesicles produced from Dodge ghosts are shown in Table IV, and data for glycol ghosts are shown in

TABLE II. Dodge Ghosts

	Percent of total protein						
	Bands 1 and 2	Bands 2.1 and 2.2	Band 3	Bands 4.1 and 4.2	Band 4.5	Band 5	Band 6
Intact ghosts							
Mean	34	10	21	5	3	5	4
SD	4.6	1.7	3.7	1.2	0.8	2.3	0.5
SEM	1.9	0.7	1.5	0.5	0.3	1.0	0.2
Sheared ghosts							
Mean	35	9	19	5	3	6	3
SD	3.7	1.0	2.9	1.8	1.6	0.8	0.5
SEM	1.5	0.4	1.2	0.7	0.7	0.3	0.2
Microvesicle classes							
Top							
Mean	45	11	22	6	5	6	3
SD	10.4	2.6	4.2	3.3	1.2	0.3	2.7
SEM	4.2	1.1	1.7	1.3	0.5	1.2	1.1
Middle							
Mean	44[a]	13	28	8	1	4	2
SD	2.6	1.0	3.3	1.0	–	0.8	1.2
SEM	1.1	0.4	1.3	0.4	–	0.3	0.5
Bottom							
Mean	60[a]	14	20	4	0	2	0
SD	14.7	4.0	10.8	4.6	–	3.0	–
SEM	6.0	4.7	4.4	1.9	–	1.2	–

[a]Difference between middle and bottom P = 0.02 by paired T analysis.

TABLE III. Glycol Ghosts

	Percent of total protein					
	Bands 1 and 2	Bands 2.1 and 2.2	Band 3	Bands 4.1 and 4.2	Band 4.5	Band 5
Intact ghosts						
Mean	35	13	21	5	3	5
SD	2.1	3.0	2.9	1.4	0.6	1.4
SEM	0.9	1.2	1.2	0.6	0.2	0.6
Sheared ghosts						
Mean	33	12	21	6	2	5
SD	4.2	2.3	1.8	1.6	1.0	1.6
SEM	1.1	0.9	0.7	0.7	0.4	0.6
Microvesicle classes						
Top						
Mean	31[c]	12	38[b]	11	0	8
SD	5.2	2.2	4.6	3.4	–	2.5
SEM	2.1	0.9	1.9	1.4	–	1.0
Middle						
Mean	45[a]	14	28	8	0	5
SD	3.4	1.9	2.0	1.1	–	0.8
SEM	1.4	0.8	0.8	0.4	–	0.3
Bottom						
Mean	53[a,c]	19	24[b]	2	0	3
SD	12.3	9.0	12.4	4.5	–	6.5
SEM	5.0	3.7	5.1	1.8	–	2.7

[a]Difference between middle and bottom $P < 0.01$.
[b]Difference between top and bottom $P < 0.05$.
[c]Difference between top and bottom $P < 0.01$
Differences calculated by paired T analysis.

Table V. Both types of ghosts show an increase in PAS-1 glycoprotein and decrease in PAS-2 glycoprotein as the vesicle classes sediment more rapidly in the gradient. PAS-3 sialoglycopeptides are missing from the bottom vesicle class produced from either type of ghost.

In order to determine if any vesicle class was either absolutely or relatively depleted or enriched with PAS staining sialoglycopeptides, identical amounts of protein were added to paired acrylamide tube gels, and then after electrophoresis one was stained with Coomassie blue and the other with PAS. Then, keeping the densitometer sensitivity fixed, the Coomassie blue stained gel and the PAS stained gel were scanned in sequence, and the protein bands were identified, cut out, and weighed. The weight ratio of PAS stained material to Coomassie blue stained material then gave an arbitrary estimate of the ratio of the amount of sialoglycoprotein to total protein. The mean values for these ratios are shown in Table VI, and there is neither enrichment nor depletion of glycoprotein in any of these fractions.

Enzyme Patterns in the Vesicle Classes

Measurement of membrane enzymes in the microvesicle classes was then undertaken using ten different normal donors in preparing hypotonic ghosts and seven different normal donors in the preparation and study of isotonic ghosts. Three experiments were also performed in which Dodge ghosts were separated on Percoll gradients; however, the results of

TABLE IV. Dodge Ghosts*

	Percent distribution of PAS-stained glycoproteins			
	PAS-1	PAS-4	PAS-2	PAS-3
Intact ghosts				
Mean	45	5	38	8
SD	9.0	2.0	5.8	1.2
SEM	3.7	0.8	2.4	0.5
Sheared ghosts				
Mean	45	4	42	8
SD	3.5	0.4	2.9	1.9
SEM	1.5	0.2	1.2	0.8
Microvesicle classes				
Top				
Mean	24	0	65	13
SD	12.0	–	17.4	7.0
SEM	4.9	–	7.0	2.4
Middle				
Mean	35	0	57	11
SD	1.8	–	4.3	1.5
SEM	0.7	–	1.7	0.6
Bottom				
Mean	95	0	5	0
SD	13.1	–	13.1	–
SEM	5.3	–	5.3	–

*Differences by paired T analysis. PAS-1: Sheared vs top, middle $P < 0.01$; middle vs top $P < 0.01$; bottom vs sheared, top, middle $P < 0.01$. PAS-4: Sheared vs top, middle, bottom $P < 0.01$. PAS-2: Sheared vs top $P < 0.05$; sheared vs middle $P < 0.01$; bottom vs sheared, top, middle $P < 0.01$. PAS-3: Sheared vs top $P < 0.05$; sheared vs middle $P < 0.01$; bottom vs sheared, top, middle $P < 0.01$.

the enzyme assays did not differ from those obtained using sucrose density gradient separation, and they are not shown. Because the different individual donors had rather different values for enzyme activity, the Friedman test was used to compare relative enzyme activities in the three vesicle classes. In this analysis, the highest activity for a given enzyme in the three microvesicle classes was recorded as 3, the next highest as 2, and the lowest activity as 1. An analysis of ranking was carried out for Dodge ghosts and derivative fractions as shown in Table VII. For example, Mg^{++}-ATPase was highest in the middle fraction, next highest in the top fraction, and lowest in the bottom fraction, and this pattern of activity is highly significant with a P value of 0.006. The comparable values for glycol ghosts are shown in Table VIII. The isotonic ghost preparation results in loss of GAPD [15]. Addition of saponin to the bottom vesicle class did not enhance the activity of AcCh (data not shown).

Since it is known that the membrane binding site for GAPD is a specific point on the band 3 molecule, the activities of GAPD were recalculated per mg of band 3, using the data given in Tables II, III, VII, and VIII. For comparative purposes the other membrane enzymes were also recalculated. The recalculated values are shown in Table IX, and the relative rank ordering of enzymes in the microvesicle classes is preserved in this recalculation, suggesting that the level of enzyme activity in a fraction depended on variables in addition to the band 3 content.

TABLE V. Glycol Ghosts*

	Percent distribution of PAS-stained glycoproteins			
	PAS-1	PAS-4	PAS-2	PAS-3
Intact ghosts				
Mean	39	2	43	12
SD	8.4	2.0	8.5	1.0
SEM	3.4	0.8	3.5	0.4
Sheared ghosts				
Mean	38	1	48	12
SD	3.2	1.3	4.6	2.0
SEM	1.3	0.5	1.9	0.8
Microvesicle classes				
Top				
Mean	20	0	66	16
SD	11.0	–	8.2	3.4
SEM	4.6	–	3.3	1.4
Middle				
Mean	30	0	59	13
SD	4.6	–	5.4	1.5
SEM	1.9	–	2.2	0.6
Bottom				
Mean	100	0	0	0
SD	–	–	–	–
SEM	–	–	–	–

*Differences by paired T analysis. PAS-1: Sheared vs top $P = 0.01$; sheared vs middle $P < 0.05$; middle vs top $P = 0.03$; bottom vs sheared, top, middle $P < 0.01$. PAS-2: Sheared vs top, middle $P < 0.01$; bottom vs sheared, top, middle $P < 0.01$. PAS-3: Bottom vs sheared, top, middle $P < 0.01$.

TABLE VI. Ratio of Sialoglycoproteins to Total Protein (Mean Values)

			Microvesicle classes		
	Intact ghosts	Sheared ghosts	Top	Middle	Bottom
Dodge ghosts	0.10	0.11	0.09	0.07	0.12
Glycol ghosts	0.08	0.09	0.15	0.08	0.10

DISCUSSION

When human erythrocyte membranes are sheared at low pressures and under circumstances where the pH, ionic strength, and temperature are controlled, the resulting vesicles are different from those produced under other circumstances. These vesicles can be separated on sucrose density gradients, partly by size but mainly by density considerations [7], into three discrete visible bands. Our prior studies showed that these vesicle classes differed with respect to their lipid content, with the top band containing significantly more lipid relative to protein [8] and the middle band containing most Mg^{++}-ATPase and Na^+,K^+-ATPase [7,8]. When these vesicle classes are studied according to more current understanding of red cell

TABLE VII. Dodge Ghosts

Enzyme	Enzyme activities of fractions		Microvesicle classes		
	Intact ghosts	Sheared ghosts	Top	Middle	Bottom
GAPD[a]					
Mean	1,600	1,623	1,102	1,270	621
SD	412	427	548	451	447
SEM	168	135	173	143	149
Ranking of activity in gradient[b]				2 = 3	1
P = 0.016					
Acetylcholinesterase[a]					
Mean	1,245	1,410	810	865	254
SD	283	656	222	416	153
SEM	116	207	70	132	51
Ranking of activity in gradient[b]				2 = 3	1
P < 0.001					
NADH oxidoreductase[a]					
Mean	7.7	6.0	3.4	5.0	6.0
SD	2.1	3.7	2.3	2.3	5.7
SEM	0.8	1.2	0.7	0.7	1.9
Ranking of activity in gradient[b]				NS	
P = NS					
Mg^{++}-ATPase[c]					
Mean	520	427	228	341	157
SD	182	164	121	219	229
SEM	69	52	38	69	76
Ranking of activity in gradient[b]			2	3	1
P = 0.006					
Na^+,K^+-ATPase[c]					
Mean	186	101	70	99	22
SD	82	68	46	64	30
SEM	31	23	14	21	10
Ranking of activity in gradient[b]			2	3	1
P = 0.01					

[a]Enzyme activity expressed as nanomoles/min/mg protein.
[b]The statistical analysis was done by Friedman's test. The highest value for an enzyme in the gradient fraction was recorded as 3, the next highest as 2, and the lowest value as 1. P indicates whether the ranking achieved statistical significance.
[c]Enzyme activity expressed as nanomoles/h/mg protein.

membrane biology [23, 24], it is clear that they do not resemble either the endocytic vacuoles [4, 5], which can be produced from white ghosts, or the exocytic vacuoles [3], which can be produced from intact red cells. Endocytic [4] and exocytic vacuoles [3] and inside-out vesicles [25, 26] are spectrin depleted, whereas all three vesicle classes produced by shearing retained relatively high concentrations of spectrin (Tables II and III). There even appeared to be relatively more spectrin than was present in parent, intact, or sheared ghosts in vesicles prepared from Dodge ghosts. However, this finding is probably artifactual and reflects in part a loss of the staining of minor bands like 4.9, 7, 4.5, and 6.

TABLE VIII. Glycol Ghosts

Enzyme	Enzyme activities of fractions		Microvesicle classes		
	Intact ghosts	Sheared ghosts	Top	Middle	Bottom
Acetylcholinesterase[a]					
Mean	1,590	1,730	660	685	281
SD	454	923	186	339	173
SEM	262	349	70	128	71
Ranking in gradient[b]			2 = 3		1
P = 0.006					
NADH oxidoreductase[a]					
Mean	6.4	4.8	2.3	3.6	7.5
SD	0.3	2.2	1.5	1.5	6.9
SEM	0.2	0.9	0.6	0.6	2.8
Ranking in gradient[b]			1 = 2		3
P = 0.002					
Mg^{++}-ATPase[c]					
Mean	852	1,152	545	764	185
SD	493	804	306	617	229
SEM	247	284	116	233	93
Ranking in gradient[b]			NS		
P = NS					
Na^+,K^+-ATPase[c]					
Mean	–	291	109	92	22
SD	–	166	99	87	28
SEM	–	63	38	30	11
Ranking in gradient[b]			NS		
P = NS					

[a]Enzyme activity expressed as nanomoles/min/mg protein.
[b]The statistical analysis was done by Friedman's test. The highest value for an enzyme in the gradient fraction was recorded as 3, the next highest as 2, and the lowest value as 1. P indicates whether the ranking achieved statistical significance.
[c]Enzyme activity expressed as nanomoles/h/mg protein.

TABLE IX. Enzyme Activities of Fractions*

Enzyme	Intact ghosts	Sheared ghosts	Microvesicle classes		
			Top	Middle	Bottom
GAPD					
Dodge ghosts	1,600/7,620	1,623/8,540	1,102/4,833	1,270/4,585	621/3,105
Glycol ghosts	–	–	–	–	–
Acetylcholinesterase					
Dodge ghosts	1,245/5,930	1,410/7,420	810/3,553	865/3,123	254/1,270
Glycol ghosts	1,590/7,570	1,730/8,240	660/1,737	685/2,362	281/1,124
NADH oxidoreductase					
Dodge ghosts	7.7/37	6.0/32	3.4/15	5.0/18	6.0/30
Glycol ghosts	6.4/30	4.8/23	2.3/6	3.6/12	7.5/30
Mg^{++}-ATPase					
Dodge ghosts	520/2,476	427/2,247	228/1,000	341/1,231	157/785
Glycol ghosts	852/4,057	1,152/5,533	545/1,434	764/2,634	185/736
Na^+,K^+-ATPase					
Dodge ghosts	186/886	101/532	70/307	99/357	22/110
Glycol ghosts	–	291/1,386	109/287	92/317	22/88

*Specific activities expressed as: sp act mg^{-1} total membrane protein/sp act mg^{-1} band 3.

The three vesicle classes are probably leaky, since external and cytosol facing enzymes can be detected in each. Furthermore, using Dodge ghosts, we had previously shown that the sialic acid in all vesicle classes was accessible to externally added neuraminidase [8]. The fact that both Dodge and glycol ghosts produce similar sorts of vesicle classes suggests that the findings are not produced by an artifact of one form of ghost preparation.

The middle vesicle class retains many of the features of intact undisrupted ghosts. Middle vesicles contain most Mg^{++}-ATPase and Na^+,K^+-ATPase, both complex enzymes that seem to require preservation of relatively large areas of membrane topography [9, 10, 27]. Middle vesicles also have a full complement of both inward- and outward-facing enzymes (Tables VII and VIII). Coomassie blue stained proteins of middle vesicles are most like those of the parent intact ghosts or sheared ghosts (Tables II and III). Sialoglycoproteins of the middle vesicles prepared from either Dodge or glycol ghosts differ from the parent sheared ghosts in showing a decrease in PAS-1 and a proportional increase in PAS-2. Minor variations in handling and processing of samples can convert PAS-1 to PAS-2 [19, 23]. The experiments were designed to minimize this sort of variation (see Methods), and therefore it appears that middle vesicles have an increased amount of glycophorin A monomer at the expense of the dimer. It is unlikely that shearing per se caused a shift from PAS-1 to PAS-2, because the sialoglycoproteins of bottom vesicles are essentially free of PAS-2 (Tables IV and V), whereas PAS patterns of sheared ghosts are identical to that of intact ghosts (Tables IV and V).

Top vesicles have relatively more lipid [8], less NADH Ox-Red (a finding that reaches statistical significance only in glycol ghosts) (Tables VII and VIII), high acetylcholinesterase (AcCh), and in Dodge ghosts high values for GAPD. Mg^{++}-ATPase and Na^+,K^+-ATPase are almost as active as they are in middle vesicles. Membrane proteins by PAGE are not strikingly different from the patterns seen in the parent sheared ghosts (Tables II and III). Sialoglycoproteins in top vesicles prepared from both Dodge and glycol ghosts show a shift from PAS-1 to PAS-2 that is even more obvious than that seen with the middle vesicles. This shift could not be due to the shearing process per se, since the PAS staining of sheared ghosts is identical to that of intact ghosts (Tables IV and V). Vesicles from the upper part of the gradient are probably enriched in glycophorin A monomer [19, 23], as represented in PAS-2. Differences in PAS-3 in top and middle vesicles are seen only in Dodge ghosts and therefore are not considered to be a reproducible finding amenable to analysis.

The bottom vesicle class is the most distinctive. When prepared from glycol ghosts it has the most NADH Ox-Red, and when prepared from Dodge ghosts it has the least GAPD (Tables VII and VIII). Irrespective of preparation, bottom vesicles generally have lowest Mg^{++}-ATPase, Na^+,K^+-ATPase, and acetylcholinesterase. PAGE analysis tends to show increased amount of spectrin (Tables II and III). Glycoprotein analysis shows a distinct enrichment of PAS-1, which presumably represents glycophorin A dimer, a decrease in PAS-2 (glycophorin A monomer), and absent PAS-3, which represents in part glycophorin B (Tables IV and V) [19, 23].

One can attempt to determine how shearing could have disrupted the membrane into vesicle classes using enzymic, protein, and carbohydrate markers. Enzymic markers have been used as indicators of external or cytosol facing proteins [25], whereas spectrin is known to be a cytosol facing protein of the cytoskeleton [24], and the PAS staining carbohydrates of glycophorin are known to extend from the external surface of the membrane [19, 23]. Middle vesicles retain enzymes reflecting outer and inner surfaces; their protein pattern is not substantially different from that of intact ghosts, although they are enriched in PAS-2, which represents in part glycophorin A monomer. However, the amount of glyco-

protein relative to total protein in any vesicle class is not consistently different from that measured in the parent ghosts. Presumably radial shearing could have produced the middle vesicle class. Top vesicles have relatively more lipid [8], but their protein content is not distinctly different from that of the parent ghosts (Tables II and III), and the total content of glycopeptides is not different from intact ghosts (Table VI). There is, however, more PAS-2, representing glycophorin A monomer. Since the enzyme patterns reflect preservation of inner and outer surfaces, a tangential cut could not have produced top vesicles. A radial cut could conceivably have produced top vesicles, but then their increase in lipids [8] relative to protein and their increase in glycophorin A monomer (PAS-2) at the expense of PAS-1 becomes difficult to explain by this analysis. Bottom vesicles could conceivably have been formed by a tangential cut that selected the inner or cytosol membrane face. Bottom vesicles have least AcCh, most NADH Ox-Red, and most spectrin; the latter two are cytosol facing proteins. However, the sialoglycopeptide content of bottom vesicles is not decreased (Table VI), and in bottom vesicles prepared from Dodge ghosts the GAPD activity, a cytosol face marker, is low. If a radial cut produced bottom vesicles, it is hard to explain their low level of Mg^{++}-ATPase, Na^+,K^+-ATPase, and acetylcholinesterase.

Since the vesicle classes differ most with respect to their sialoglycopeptide distribution, it is conceivable that shearing occurred at repeating mechanically weak spots in the membrane, which are marked by patterns of sialoglycopeptide assembly.

On the basis of this sort of analysis it is highly unlikely that shearing produced tangential cuts through the membrane allowing inner and outer face membrane units to separate and then to reseal themselves into vesicles. Radial shearing is more likely, but radial shearing through a pure fluid-mosaic membrane cannot easily account either for the fact that three distinct vesicle classes were produced or for the differences seen in the three vesicle classes. The results are more consistent with radial shearing occurring through a membrane where relatively fixed domains were superimposed on a fluid-mosaic model. Marchesi's models of a fixed matrix [2] occurring periodically within a fluid-mosaic background is most consistent with our observations and is supported by experiments exploring the linkage of the cytoskeleton of the erythrocyte membrane to integral proteins [24, 28]. The shearing procedure may have, in a manner analogous to freeze-cleaving, sought out mechanical weaknesses in the membrane perhaps marked by sialoglycopeptides, which allowed for the breaking up of the intact ghost into a series of classes that reflect the fixed matrix aspect of the membrane.

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