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CHARACTERIZATION OF MICROVESICLES PRODUCED BY SHEARING OF HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

Human erythrocyte membranes were sheared into microvesicles with substantial loss of activity of the ATPases but not the nitrophenyl phosphatases. This loss of ATPase activity was not accompanied by alteration in lipid content, sialic acid content or orientation, or sulfhydryl group reactivity. Centrifugation in a linear sucrose gradient, resolved the microsvesicles into distinct classes which differed with respect to their lipid content, the reactivity of their sulfhydryl groups, and the activity of their ATPases and nitrophenyl phosphatases. Vesicles with low sulfhydryl reactivity had low activity of all enzymes tested. Vesicles containing relatively high lipid to protein and lipid to sialic acid ratios had low ATPase and K^+-dependent nitrophenyl phosphatase activities. We have found that membranes could be sheared into readily separable membrane classes containing varying amounts of lipid relative to sialic acid and protein, and this observation supports a segmental concept of membrane structure. The parallelism of (Na^+, K^+)-ATPase and K^+-dependent nitrophenyl phosphatase activities in the separated microvesicle classes stands in contrast to their divergent behavior following shearing where the K^+-dependent nitrophenyl phosphatase is not inactivated. This pattern suggested that K^+-dependent nitrophenyl phosphatase probably accurately reflected the K^+-dependent dephosphorylation phase of (Na^+, K^+)-ATPase; however, the prior step required a higher degree of structural integrity and was very susceptible to inactivation by chemical or mechanical perturbation.

INTRODUCTION

In studies directed toward probing the relationship of erythrocyte membrane structure with the biologically important membrane-bound enzyme, (Na^+, K^+)-ATPase, we had previously shown that human erythrocyte membranes could be disrupted into microvesicles by shearing in a French pressure cell[1]. Centrifugation in a sucrose gradient resolved these microvesicles into at least two distinct and widely separated classes which differed with respect to their content of (Na^+, K^+)-ATPase and Mg^{2+}-ATPase. The microvesicles with greater specific activity of (Na^+, K^+)-ATPase contained more trilamellar structure as visualized by electron microscopy.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

The fact that membranes could be disrupted into distinct classes of vesicles tended to support the concept of heterogeneity in membrane structure with shearing occuring at structurally weak repeating loci.

This report deals with the chemical differences in the microvesicle classes which might lead to their separability in the gradient because such difference could bear on the nature of the hypothetical membrane subunits. The vesicles were accordingly subjected to direct analysis for lipid, protein, sialic acid and sulfhydryl content.

The ability to separate microvesicle classes which differed in their ATPase activity also provided an opportunity to test the positive correlations which have been proposed for (Na^+, K^+)-ATPase on one hand and K^+-dependent nitrophenyl phosphatase[2], membrane sulfhydryl[3] groups and phospholipid content[4,5] on the other.

MATERIALS

Neuraminidase was obtained from Worthington Laboratories. ^{14}C-labeled *p*-chloromercuribenzoate ([^{14}C]PCMB) was obtained from Schwartz Bio Research, Inc. and had a specific activity of 12 mC/mmole. Other materials utilized were obtained as previously described[1].

METHODS

Human erythrocyte membranes were prepared from blood stored in acid–citrate–dextrose and no difference was observed between blood stored for 2–21 days. Membranes were prepared by stepwise hypotonic lysis as previously described[1] with two modifications: bovine serum albumin was not added to the last two steps of the lysis, and in the last step 10 mM Tris–HCl buffer (pH 7.4) was substituted for 154 mM NaCl.

Membranes were stored frozen at −20° for no more than 3 weeks, and prior to use, membranes were suspended in a medium of 30 % sucrose, 5 mM Tris–HCl buffer (pH 7.0) and 3 mM $MgCl_2$ and were then sheared in a prechilled Aminco French Press with a 2.56-inch ram at a hydraulic pressure setting of 500 lb/inch^2. The unsheared particles were removed by centrifugation[1] and approx. 2.5 mg of membrane protein was layered on a 30–43 % linear sucrose gradient (monitored by refractometry) and centrifuged at 4°, 284000 × *g* in a Spinco SW-40 rotor for 3 h. Each of the six tubes utilized had a heavy visible band of material near the bottom and a lighter visible band just beneath the meniscus (Fig. 1). The major lowermost band generally appeared in a sucrose density of 1.15 while the uppermost band appeared at a sucrose density of 1.12. Equilibrium was not achieved after 3 h of centrifugation. When centrifugation was carried out to equilibrium at 20 h, both bands were unchanged in position. However, the protein content of the uppermost band had decreased by half. Therefore, the separation achieved in 3 h represents a combination of velocity sedimentation and differences in buoyant density of the vesicles. The 12.5 ml of the gradient was resolved into approx. 30 fractions containing 6 drops each, and parallel fractions from each of the six tubes were pooled and the protein content of each fraction was determined by the method of LOWRY *et al.*[6].

ATPase was assayed by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP by enzyme action in the presence of Mg^{2+}, or Mg^{2+}, Na^+ and K^+ (ref. 1). Protein content was

determined and the ATPase activity was expressed as μmoles P_i per h per mg membrane protein.

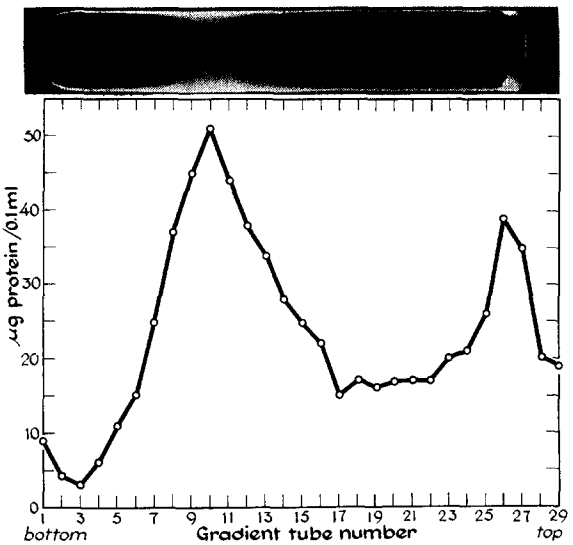

Fig. 1. Composite picture showing the actual gradient tube above, with the two major visible bands, and below the protein concentration of the gradient fractions.

Nitrophenylphosphatase was measured by a modification of the original method of FUJITA *et al.*[7] which has been described elsewhere[8]. Because of its low specific activity in erythrocyte membranes, membrane material was concentrated as follows: 9 vol. of 3 mM $MgCl_2$ were added to appropriate fractions of the gradients in order to reduce sucrose concentration and the suspension was centrifuged at 4°, 250000 × *g* for 150 min. The membrane pellet was resuspended in a small volume of water, and aliquots were taken for protein determination and sialic acid measurement. Adequate amounts of membrane protein were now available for nitrophenylphosphatase assay which was performed in the presence and absence of K^+, the difference representing K^+-dependent nitrophenylphosphatase. The results were expressed as nmoles *p*-nitrophenol liberated per h per mg protein.

Total lipid, cholesterol and phospholipid analyses were performed on whole, sheared and separated membrane vesicles using chloroform–methanol (2:1, by vol.) extraction[9]. After separation into two phases, the chloroform extract was carefully isolated and total lipids determined gravimetrically. Water-pumped N_2 was used to evaporate the solvent, and care was taken in subsequent handling of lipids to prevent oxidation.

Since sucrose was found to be present in the chloroform extracts, the residue was dissolved in petroleum ether, centrifuged from insoluble material, and reevaporated to give a more accurate total lipid value. A control extract prepared from 1 ml saturated sucrose solution yielded a net residual weight of 1.5 mg.

The lipids were then redissolved in chloroform and small aliquots taken for total phospholipid analysis by the method of BARTLETT[10]. The individual phospholipids were separated by thin-layer chromatography on Brinkman Silica Gel HR (0.75 mm thick coating) with chloroform–methanol–glacial acetic acid–water

(65:25:8:4, by vol.) and were visualized by a brief exposure to I_2 vapors. Commercial phospholipids were used for identification. The silica gel zones containing phospholipids were scraped directly into Pyrex tubes, digested with H_2SO_4 and analyzed for phosphate by the method of PARKER AND PETERSON[11].

Cholesterol was isolated by elution of the free cholesterol zone on the thin-layer chromatographic plate with diethyl ether and determined by the $FeCl_3$ color reaction[12].

The sum of the cholesterol *plus* phospholipids corresponded to about 75 % of the total lipids determined gravimetrically except when the amount of lipids in the whole fraction was very small. Since the phospholipid and cholesterol analyses were more accurate than the gravimetric total lipid values, the data reported in Table II include only cholesterol, phospholipids and their sum.

Sialic acid was determined by the method of WARREN[13] following either H_2SO_4 digestion or neuraminidase attack. The latter was performed exactly as described by STECK *et al.*[14].

The sulfhydryl content of membranes was estimated by measuring the amount of [^{14}C]PCMB bound to the membrane fractions. The protein content of aliquots of whole and sheared membranes as well as gradient fractions was determined and 100–300 nmoles of PCMB were added per mg of membrane protein. The suspension was incubated at room temperature for 30 min after which it was centrifuged at 90000 × *g*, 4° for 150 min and the membrane pellet was subsequently resuspended and washed 3 times in a 50-fold volume of 10 mM $MgCl_2$. The pellet was then suspended in a known volume, protein content was determined, and an aliquot counted in Bray's solution using a liquid scintillation spectrometer with 60 % efficiency for ^{14}C. The results were expressed as nmoles of PCMB bound per mg of membrane protein. Our estimate of 40 nmoles of externally oriented sulfhydryl per mg membrane protein is in substantiala greement with the recalculated data of REGA *et al.*[15], who used *p*-chloromercuribenzene sulfonate, a reagent which penetrates the membrane even less than PCMB. For the gradient fractions, it was necessary to remove the sucrose in order to sediment membrane vesicles as pellets from aqueous medium. Therefore, following addition of the calculated amount of PCMB and incubation, the suspension was dialyzed overnight in the cold against 10 mM $MgCl_2$. Subsequently, the washing and measurement of bound PCMB were as described above.

RESULTS

Comparison of whole and sheared membranes

We had previously shown that the shearing procedure resulted in a mean loss of 40 % of ATPase activity involving Mg^{2+}-ATPase and (Na^+, K^+)-ATPase almost equally[1]. The effects of shearing on ATPase activity and nitrophenylphosphatase activity were therefore compared (Table I). The mean loss of Mg^{2+}-ATPase following shearing was 32 % while loss of (Na^+, K^+)-ATPase was 43 %, and both losses are significant at the 0.05 level. In contrast, there was no significant loss of activity of either K^+-independent or K^+-dependent nitrophenylphosphatase following shearing.

Since it was recently shown that another method of preparing membrane microvesicles resulted in some inside-out vesicles[14], membrane sialic acid content was measured using digestion with H_2SO_4, which releases total sialic acid, and neuraminidase attack, which releases only externally oriented sialic acid (Table I).

Shearing did not alter the overall sialic acid content of membranes and the amount of sialic acid released by H_2SO_4 digestion was comparable to that released by neuraminidase attack. Membrane sulfhydryl groups available for binding to PCMB were equal in whole and sheared membrane (Table I).

TABLE I

COMPARISON OF WHOLE AND SHEARED MEMBRANES

Results expressed as mean ± standard deviation. The number in parentheses indicates the number of experiments performed.

	Whole membranes	*Sheared membranes*
ATPase (μmoles P_i/h per mg protein)		
Mg^{2+}-ATPase	0.69 ± 0.26 (8)	0.47 ± 0.14 (9)
(Na^+, K^+)-ATPase	0.42 ± 0.17 (8)	0.24 ± 0.12 (9)
Nitrophenylphosphatase (nmoles *p*-nitrophenol/h per mg protein)		
K^+-independent nitrophenylphosphatase	30 ± 6 (7)	31 ± 5 (9)
K^+-dependent nitrophenylphosphatase	20 ± 5 (6)	17 ± 4 (9)
Sialic acid content of membranes (μmoles/mg protein)		
H_2SO_4 digestion	0.103 ± 0.020 (7)	0.109 ± 0.013 (9)
Neuraminidase digestion	0.094 ± 0.026 (4)	0.101 ± 0.018 (5)
Sulfhydryl groups titrated with [^{14}C]PCMB (nmoles/mg protein)	39 ± 12 (7)	39 ± 7 (8)

Properties of the microvesicle classes

Under the conditions described the sheared microvesicles were visibly resolved in the gradient into two major classes. Each gradient was distributed into approx. 30 fractions which were numbered in sequence from the bottom of the gradient, and the protein content of each fraction was determined and plotted (Fig. 1). The material closest to the bottom of the tube appearing in Fractions 5–7 was termed the leading edge, Fractions 9–11 were termed the peak, and the material in Fractions 24–27 was called the top.

Correlation of lipid content with ATPase activity of the membrane microvesicle classes. Following the separation of microvesicles on the sucrose gradient, aliquots of pooled segments of the gradient were assayed for ATPase activity as well as lipid content (Table II). The microvesicle class near the bottom of the tube (leading edge) had lower lipid to protein ratio, and this observation was confirmed in four subsequent experiments. The peak fraction contained greatest activity of (Na^+, K^+)-ATPase but did not have the highest lipid to protein ratio or phospholipid to protein ratio. Phosphatidyl ethanolamine and phosphatidyl serine were not found in greater proportions in the peak portion of the gradient (Table II). The apparent increase in ratio of cholesterol to phospholipid seen in the top vesicle portion was not confirmed in two subsequent experiments where the ratio of cholesterol to phospholipid was 0.58–0.62 in all fractions.

Measurement of sialic acid content of microvesicles. Sialic acid, which is externally oriented in intact red blood cells provides a useful index of externally oriented membrane segments. The shearing procedure could have sheared membranes tangentially

TABLE II

LIPID COMPOSITION OF WHOLE AND SHEARED MEMBRANES

Membrane fraction tested	*Mg^{2+}-ATPase**	*(Na^+, K^+)-ATPase**	*Ratios*			*Phospholipid distribution (%)***					
			*Lipid*** / *Protein*	*Cholesterol / Phospholipid*	*Phospholipid / Protein*	*PE*	*PS*	*PC*	*Sph*	*Lyso*	*P.O.*
Whole membranes	0.68**	0.44	1.03	0.54	0.69	26.4	13.9	32.4	22.9	1.7	2.7
Sheared membranes	0.42	0.11	0.94	0.53	0.61	23.7	14.5	33.1	26.0	2.0	0.7
Leading edge gradient	0.24	0.12	0.74	0.54	0.48	26.2	15.0	33.1	22.1	3.0	0.6
Peak gradient	0.29	0.21	0.87	0.52	0.57	24.1	14.0	33.0	25.6	3.1	0.2
Top gradient	0.14	0.09	1.22	0.60	0.76	24.8	13.1	31.7	26.1	3.5	0

* ATPase activities expressed as in Table I.

** Lipid refers to the sum of cholesterol and phospholipid (see METHODS).

Abbreviations: PE, phosphatidyl ethanolamine; PS, phosphatidyl serine; PC, phosphatidyl choline; Lyso, lysolecithin; P.O., point of origin; Sph, spingomyelin.

TABLE III

CHARACTERIZATION OF MICROVESICLE CLASSES

The units and symbols used are exactly as described in Table I.

	Sheared membranes	*Gradient fractions*		
		Leading edge	*Peak*	*Top*
Mg^{2+}-ATPase	0.47 ± 0.14 (9)	0.20 ± 0.10 (8)	0.44 ± 0.23 (9)	0.14 ± 0.07 (9)
(Na^+, K^+)-ATPase	0.23 ± 0.12 (9)	0.10 ± 0.05 (8)	0.29 ± 0.14 (9)	0.12 ± 0.06 (9)
K^+-independent nitrophenylphosphatase	31 ± 5 (9)	23 ± 8 (6)	31 ± 5 (7)	29 ± 5 (5)
K^+-dependent nitrophenylphosphatase	17 ± 4 (9)	9 ± 4 (6)	17 ± 2 (7)	9 ± 4 (5)
Sialic acid content				
H_2SO_4 digestion	0.109 ± 0.013 (9)	0.100 ± 0.010 (8)	0.108 ± 0.015 (8)	0.100 ± 0.014 (6)
Neuraminidase digestion	0.101 ± 0.018 (5)	0.080 ± 0.015 (4)	0.096 ± 0.004 (4)	0.084 ± 0.011 (2)

thereby resulting in vesicles relatively enriched with segments from the outermost or innermost portions of the ghost membrane. Determination of total sialic acid after H_2SO_4 digestion, would indicate if shearing had resulted in selective dissociation between innermost and outermost membrane segments. Furthermore, if shearing resulted in membrane vesicles being turned "inside out", there would be a measurable difference between sialic acid released by neuraminidase attack as contrasted to H_2SO_4 digestion. All microvesicles contained the same amount of sialic acid relative to protein content and the neuraminidase attack yielded almost as much sialic acid as did H_2SO_4 digestion (Table III).

Correlation of ATPase with nitrophenylphosphatase. Because the K^+-dependent nitrophenylphosphatase has been shown to parallel, in part, the K^+-dependent dephosphorylation step of (Na^+, K^+)-ATPase, experiments comparing the activity of ATPase and nitrophenylphosphatase were performed (Table III). The microvesicles from the peak of the gradient contained highest Mg^{2+}-ATPase and (Na^+, K^+)-ATPase activities, while both the top and leading edge of the gradient consisted of vesicles

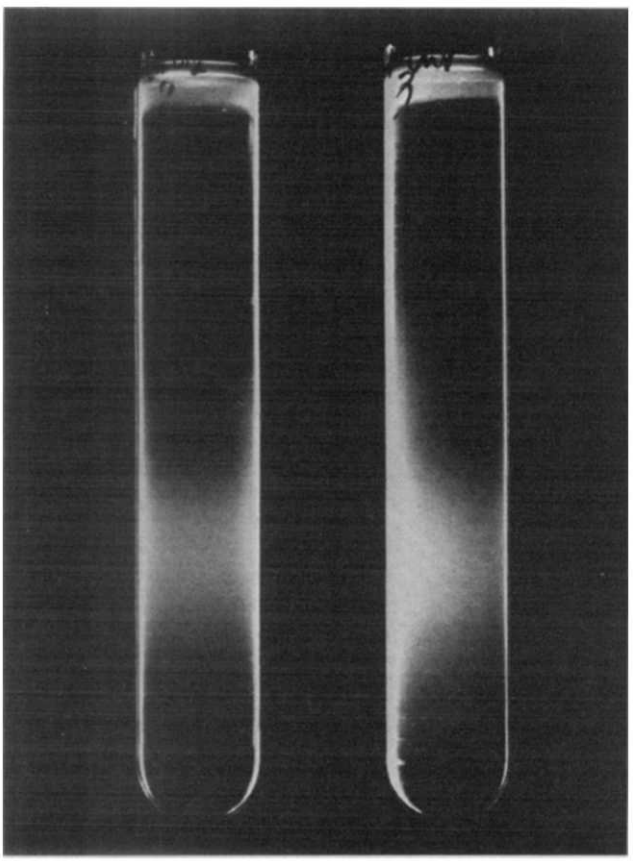

Fig. 2. Comparison of sheared vesicle bands following centrifugation in the sucrose gradients. The tube on the right contained vesicles which had been exposed to neuraminidase while the tube on the left contained sheared vesicles which had been handled identically lacking only neuraminidase treatment. No difference in pattern was apparent.

containing low Mg^{2+}-ATPase and low (Na^+, K^+)-ATPase. K^+-dependent nitrophenylphosphatase in general paralleled the ATPase activity, particularly the (Na^+, K^+)-ATPase, while the K^+-independent nitrophenylphosphatase did not. Only the leading edge vesicles showed a significant ($P = 0.05$) decrease in K^+-independent nitrophenylphosphatase.

Role of sialic acid in determining the position of vesicles in the gradient. Variation in degree of hydration of sialic acid groups could contribute to the apparent density of the vesicles[16]. Therefore, the following experiment was performed in order to assess the contribution of sialic acid composition to the determination of the position of vesicles in the gradient. Whole membranes were incubated with Ca^{2+} alone or Ca^{2+} *plus* neuraminidase under conditions described above which regularly resulted in the release of 80–93 % of the total sialic acid into the bulk phase of the medium. The suspension was washed in 10 mM Tris–HCl (pH 7.0), sheared and placed on the gradient as usual. Fig. 2. indicates that no difference in position of bands in the gradient was induced by neuraminidase attack.

Sulfhydryl content of microvesicle classes. Because ATPases have been shown to be very susceptible to inhibition by sulfhydryl inhibitors[2,3], the sulfhydryl groups available for titration with PCMB were determined for each vesicle class. The results of three experiments are listed in Table IV. While there was variation in absolute amount of PCMB bound in the individual experiments, in each case the "leading edge" vesicles contained the least accessible sulfhydryl groups.

TABLE IV

SULFHYDRYL CONTENT OF MICROVESICLE CLASSES

Sulfhydryl content is expressed as nmoles/mg protein.

Expt.	*Gradient fractions*		
	Leading edge	*Peak*	*Top*
1	32	41	46
2	21	34	30
3	22	29	28

DISCUSSION

Shearing of membranes resulted in a 30–40 % decrease in specific activity of the erythrocyte membrane ATPases in this study as it did in our prior report[1]. In order to investigate the cause of this loss of activity, measurements were made of membrane lipid, sialic acid, and titratable sulfhydryl groups before and after shearing. Passage through the French press did not result in loss of sialic acid (Table I), nor were membrane vesicles turned inside out, since equivalent amounts of sialic acid were liberated from whole and sheared membranes by H_2SO_4 digestion and neuraminidase attack. Substantial loss of lipid was not a consequence of shearing (Table II) and was therefore not a factor in the observed loss in ATPase activity. PCMB penetrates membranes very slowly and therefore exhibits considerable selectivity for sulfhydryls at the outer surface of the membrane[15]. By the [^{14}C]PCMB method, whole and sheared membranes had equivalent amounts of externally oriented sulfhydryl groups.

It has been proposed that the K^+-dependent nitrophenylphosphatase activity reflects the terminal K^+-dependent dephosphorylation phase in the hydrolysis of ATP by (Na^+, K^+)-ATPase, the initial step being a Na^+-dependent phosphorylation of a specific membrane receptor[5,17]. Nevertheless, some investigators have questioned the identity of (Na^+, K^+)-ATPase and K^+-dependent nitrophenylphosphatase[2]. In our studies, the shearing procedure resulted in no decrease in K^+-dependent nitrophenylphosphatase while (Na^+, K^+)-ATPase underwent a 40 % inhibition. It could therefore be argued that K^+-dependent nitrophenylphosphatase and (Na^+, K^+)-ATPase do not represent differing activities of a single enzyme system[5]. Alternatively the shearing process may have disrupted the Na^+-dependent transfer of phosphate from ATP to a specific membrane site. This apparent selectivety of shearing for attack on the Na^+-dependent phosphorylation step of (Na^+, K^+)-ATPase is reminiscent of the effects of carbodiimide modification of human erythrocyte membranes[8]. Carbodiimide activation of membrane carboxyl groups with subsequent cross-linking to adjacent membrane nucleophiles, results in a membrane modification that produces greater inhibition of (Na^+, K^+)-ATPase than K^+-dependent nitrophenylphosphatase. In unpublished studies[18], we have observed a similar divergence in susceptibility of (Na^+, K^+)-ATPase and K^+-dependent nitrophenylphosphatase to sulfhydryl modification. It is therefore conceivable that the Na^+-dependent phosphorylation step of (Na^+, K^+)-ATPase or some step prior to the K^+-dependent dephosphorylation, requires a very high order of membrane organization and that minor structural alterations, induced either chemically or mechanically, could result in selective inhibition of this activity.

It should be emphasized that the vesicles which are produced by shearing are the resultant of two phenomena, namely, the disruption induced by the shearing force and the subsequent annealing of membrane material into microvesicles. Attempts to dissociate and study these two phases of microvesicle formation were unsuccessful. Low pressure shearing of membranes, therefore did not alter overall lipid–protein relationships and did not sleectively remove or reverse the orientation of externally arranged sulfhydryl groups or sialic acid components. The fact that ATPases, but not nitrophenylphosphatases, were affected, suggested that the shearing resulted in a very selective perturbation or structural rearrangement of membrane components.

We had previously proposed that the shearing of membranes into distinct microvesicle classes supported those hypotheses of membrane structure which contained the concept of heterogeneity of the structural components[1,19]. If one makes several simplifying assumptions, namely that the erythrocyte membrane is spherical, that the thickness of the microvesicles is uniform and similar to the thickness of the erythrocyte membrane and that spherical microvesicles have a diameter of approximately 600Å (ref. 1) it can be calculated that each erythrocyte membrane is sheared into 10000–20000 microvesicles. Since each erythrocyte membrane has been estimated to contain approx. 200 (Na^+, K^+)-ATPase sites[20], it was conceivable that the large number of vesicles, when separated into classes, might reflect some of this enzymic and structural heterogeneity. The position of each class in the gradient could provide some information on its structure. Previous studies[16] have indicated that the separation of membrane microvesicles in a gradient composed of impermeant materials, depends on osmotic considerations as well as vesicle density and charge density. In cases, where as in our experiments, the isopycnic point is not reached, vesicle size is also a factor.

In summarizing the characteristics of the vesicle classes, the following results were obtained:

Leading edge microvesicles had low sulfhydryl content, symmetrical reduction of total lipid relative to whole or sheared membranes, normal sialic acid content, low ATPase and K^+-dependent nitrophenylphosphatase (Table III), and uniquely, low nonspecific K^+-independent nitrophenylphosphatase ($P = 0.05$ by Student's t test). The loss of lipid and sulfhydryl groups as well as decrease in K^+-independent nitrophenylphosphatase suggested that denatured vesicles were present in this class. Low lipid content certainly contributed to this class's position in the gradient.

Peak microvesicles were previously shown to have most trilamellar appearance on electron microscopy[1] and had most Mg^{2+}-ATPase and (Na^+, K^+)-ATPase. Their position in the gradient probably reflects protein–lipid relationships.

The topmost vesicle class was widely separated in the gradient from the other two classes and its position was presumably determined by its increased lipid content. Despite the established positive correlation between ATPase and phospholipid content, in particular phosphatidyl serine[4], the ATPases and K^+-dependent nitrophenylphosphatase were very low in this vesicle class. Presumably the variation in phospholipid content noted in the vesicle classes was not at a level[4] where differences in enzyme activity occur.

Sialic acid content of the vesicle classes or hydration of sialic acid anionic components did not contribute to the position of vesicle classes in the sucrose gradient (Fig. 2).

Other factors play a role in determining vesicle position in the gradient and the striking effects induced by minor variations in pH and divalent cation concentration are under study as is the behavior of these vesicles in other impermeant solutes.

There were both similarities and differences in the behavior of (Na^+, K^+)-ATPase and K^+-dependent nitrophenylphosphatase. K^+-dependent nitrophenylphosphatase was resistant to a shearing procedure which induced a 40 % loss of (Na^+, K^+)-ATPase, however, K^+-dependent nitrophenylphosphatase essentially paralleled ATPase activity in the vesicle classes. Apparently, the structural requirements were less for the K^+-dependent nitrophenylphosphatase than for either Mg^{2+}-ATPase or (Na^+, K^+)-ATPase; however, once disruption had occurred and vesicles had formed, those particles containing ATPase also contained K^+-dependent nitrophenylphosphatase in similar proportions.

The presence of equal amounts of sialic acid relative to membrane protein in all vesicle classes suggested that the shearing was radial with equivalent representation of the outermost membrane glycoprotein moieties in each class. MURPHY[19] had reported that cholesterol was concentrated about the convexity of the membrane surface. However, cholesterol and phospholipids behaved as a single unit with respect to shearing and separation into vesicle classes. This observation also suggested that shearing occurred in a radial plane. Alternatively, heterogeneity of cholesterol and phospholipid may have been present initially, but during the annealing phase of microvesicle formation, phospholipid and cholesterol may have come together in more usual ratios. Shearing, however, was able to dissociate sialic acid from membrane lipid components, since vesicles from the top of the gradient contained approx. 65 % more lipid per sialic acid moiety than did leading edge vesicles. Vesicles with the composition of the topmost class had striking reduction in specific activity of ATPase and

K^+-dependent nitrophenylphosphatase, but not K^+-independent nitrophenylphosphatase. Again, it was possible that the shearing had produced a vesicle containing less ATPase or that the annealing had produced a vesicle wherein undefined internal strains were unfavorable for ATPase activity.

The variations in lipid to protein ratios as exemplified by the peak and top microvesicle classes, suggested that shearing could produce protein segments containing approximately one or two complements of lipid. Therefore these studies tend to support those hypotheses of membrane structure which are based on the concept of mosaic or segmental arrangement of proteins alternating with phospholipid components[21,22].

Vesicles which contained (Na^+, K^+)-ATPase also contained K^+-dependent nitrophenylphosphatase; however, procedures which tended to inactivate (Na^+, K^+)-ATPase left K^+-dependent nitrophenylphosphatase relatively unaffected. Therefore, we propose that K^+-dependent nitrophenylphosphatase is part of the (Na^+, K^+)-ATPase system, and that the greater structural complexity characterizing (Na^+, K^+)-ATPase involves the steps prior to the K^+-dependent dephosphorylation of the specific membrane site.

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