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STUDIES ON ATPase IN SHEARED MICRO VESICLES OF HUMAN ERYTHROCYTE MEMBRANES*

STANLEY L. SCHRIER**, ELDAD GIBERMAN, DAVID DANON AND EPHRAIM KATCHALSKI

** *Stanford University School of Medicine, Stanford, Calif. (U.S.A.) and Weizmann Institute of Science, Rehovoth (Israel)*

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SUMMARY

Human erythrocyte membranes were disrupted by shearing in the French press. The resulting membrane vesicles could be separated by sucrose density centrifugation into 2–3 distinct classes which differed with respect to their content of (Na^+-K^+)-activated ATPase and Mg^{2+}-activated ATPase. Relatively high specific activities of (Na^+-K^+)-activated ATPase were found in the class of vesicles that retained most trilamellar structure as visualized by electron microscopy. Furthermore, the (Na^+-K^+)-activated ATPase of the separated vesicles was not completely inhibited by concentrations of oligomycin that completely inhibited (Na^+-K^+)-activated ATPase of unseparated membrane particles. It is proposed that membranes sheared at relatively low pressures are cleaved at structurally weak loci. The resulting vesicles have different proportions of (Na^+-K^+)-activated ATPase and Mg^{2+}-activated ATPase and have probably lost a binding site for oligomycin.

INTRODUCTION

A relationship between the membrane bound (Na^+-K^+)-activated ATPase and the active transport of Na^+ and K^+ has been proposed for several tissues[1,2]. Because of the biological importance of this enzyme system, attempts have been made to solubilize and study it in order to answer a series of key questions involving: the relationship of the (Na^+-K^+)-activated enzyme with the Mg^{2+}-activated ATPase, the mechanism of (Na^+-K^+)-activation and the localization of the ATPase in the membrane. Thus far solubilization of the erythrocyte membrane has resulted in irreversible loss of both Mg^{2+}-activated ATPase and (Na^+-K^+)-activated ATPase[3] (L. GOLDSTEIN, E. GIBERMAN AND S. SCHRIER, unpublished observations). In order to attack these problems, we have resorted to disruption of membrane fragments into vesicles by shear force. Using these membrane vesicles, problems of accessibility of substrate to enzyme were minimized. Furthermore, the membrane vesicles could be separated into several distinct classes which differed consistently in their content of (Na^+-K^+)-

* Request for reprints should be addressed to Stanley L. Schrier, M.D. Division of Hematology, Stanford University School of Medicine, Stanford, Calif. 94305, U.S.A.

activated ATPase and Mg^{2+}-activated ATPase and in the ability of oligomycin to inhibit the (Na^+-K^+)-activated ATPase.

MATERIALS

Sodium and Tris·ATP and oligomycin were obtained from Sigma Chemical Co. [γ-^{32}P]ATP was prepared by the method of AVRON[4]. Resulting specific activities averaged $2 \cdot 10^7$ counts/min per μmole.

METHODS

Human erythrocyte membranes were prepared as previously described[5]. The protein content of membranes was measured by the method of LOWRY *et al.*[6] and appropriate blanks were run to correct for the effect of sucrose[7]. ATPase was assayed colorimetrically[5] by a slight modification of the method of POST *et al.*[1] and isotopically[8]. In the latter method, which is approx. 50 times more sensitive than colorimetry, $^{32}P_i$ released from [γ^{32}P]ATP by enzyme action is trapped quantitatively as the phosphomolybdate complex, extracted quantitatively in isobutanol–benzene[8] and counted. In each case, assays were performed under two sets of conditions. For the colorimetric assay total ATPase was measured in a 3.0-ml volume in the presence of 0.5–1.0 mg of membrane protein, 2–3 mM ATP, 3 mM $MgCl_2$, 22 mM Tris buffer or imidazole–glycylglycine buffer (pH 7.5), 80 mM NaCl and 33 mM KCl, while Mg^{2+}-activated ATPase was measured in the absence of Na^+ and K^+. The difference between total ATPase and Mg^{2+} ATPase is (Na^+-K^+)-activated ATPase. The isotopic assay was carried out in a 1.0-ml volume using 30–150 μg of membrane protein, 0.3–1.0 mM ATP with sufficient [γ-^{32}P]ATP added to yield a final specific activity of 150000–500000 counts/min per μmole. The concentrations of Na^+-K^+, Mg^{2+} and buffer were otherwise unchanged. ATPase activity is expressed as μmoles P_i per h per mg protein. Membranes suspended in a medium of 5 mM Tris–HCl buffer (pH 7.0) and 3 mM $MgCl_2$ were sheared in prechilled Aminco French press at a dial setting of 500 lb/inch^2 to 5000 lb/inch^2 on the hydraulic press. Since the piston has a diameter of 2.56 inches the actual pressures are approx. 3000–30000 lb/inch^2. The grossly turbid membrane suspensions became translucent. The unsheared particles were removed by centrifugation at 13000 $\times$ *g* for 10 min at 4°. The resulting vesicles remained in suspension following centrifugation at 20000 $\times$ *g* for 30 min, but could be sedimented by centrifugation in the ultracentrifuge (see below). When preparing membranes for density gradient centrifugation, 30% sucrose was added to the medium prior to shearing.

The sheared membranes, in 30% sucrose, were placed on 30–45% linear sucrose gradients and centrifuged at 39000 rev./min for 5–6 h in the Spinco SW-39 rotor. Two to three visible bands appeared, and they were separated in a pressure drop collecting device. Determination of ATPase, protein content and electron microscopic appearance were performed on the separated fractions. Oligomycin was dissolved in ethanol and used at a final concentration of 60 μg/ml. Equivalent amounts of ethanol were added to control tubes and had no effect on enzyme activities.

For electron microscopy the membrane fragments from the sucrose gradient were washed free of sucrose with 3 mM $MgCl_2$. The membrane pellets were then fixed

in 1 % OsO_4 in 3 mM $MgCl_2$. Dehydration and embedding were carried out according to the method of RYTER AND KELLENBERGER[9]. Grey to silver sections were mounted on uncoated or formvar coated copper grids. After staining with uranyl acetate, sections were coated with a thin layer of carbon.

RESULTS

Membranes were disrupted by shearing under a variety of pressures in a pre-cooled French press, and the ATPase activities of the resulting particles were determined (Table I). Progressive increments in pressure led to increasing losses of ATPase activity (Table I, Expts. 1 and 2) with essentially equivalent loss of Mg^{2+}-activated ATPase and (Na^+-K^+)-activated ATPase. At pressures of 500 lb/inch^2 there was a mean loss of 35 % specific activity of total ATPase, but the ratio of Mg^{2+}-activated ATPase to (Na^+-K^+)-activated ATPase did not vary in any predictable manner. Addition of 3 mM $MgCl_2$ to the membrane suspension prior to shearing resulted in 10–20 % higher specific activity of all ATPase activities. Addition of 3 mM ATP, or 3 mM ATP in combination with 3 mM $MgCl_2$ did not result in higher ATPase activity. Shearing in 1 M KCl led to further loss of total ATPase.

The shearing process converted the membranes into vesicles of various sizes; the largest cross sectional diameter of the vesicles was approx. 0.6 μ as compared to almost 8 μ for the cross sectional diameter of a whole red blood cell (Fig. 1). Vesicles could not be sedimented by centrifugation at 20000 $\times$ g for 30 min, but centrifugation in aqueous media in the SW 39 rotor at 175000 $\times$ g for 30 min deposited 63 % of the added membrane protein in the pellet (Table II) as well as 82 % of the ATPase. Concomitantly, there was a slight increase in the ATPase specific activity in the pellet and a decrease of specific activity in the residual supernatant solution.

Since the sheared membrane vesicles seemed to vary widely in size, attempts were made to separate the vesicles and assay their ATPase. Preliminary experiments

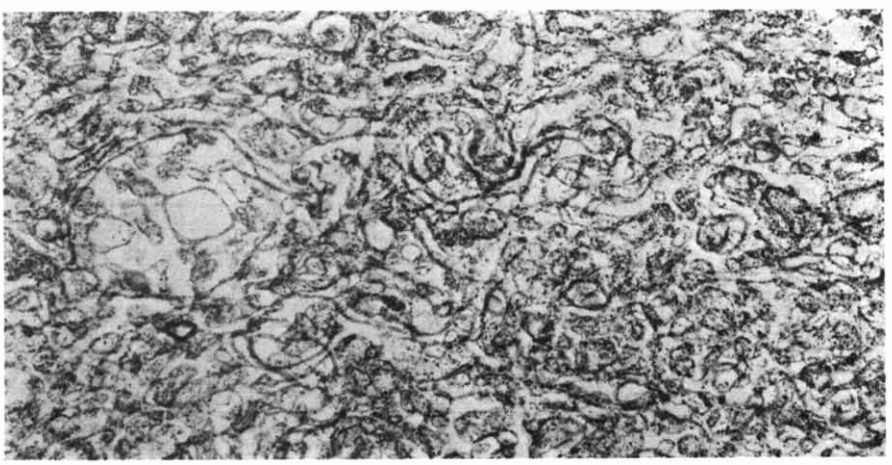

Fig. 1. Erythrocyte membranes were sheared at a dial setting of 500 lb/inch^2 in 3 mM $MgCl_2$. The unsheared particles were removed by centrifugation for 10 min at 13000 $\times$ g. The resulting supernatant solution was centrifuged at 175000 $\times$ g for 60 min and the resulting pellet was then fixed and prepared for electron microscopy as described above. Magnification = 30000. The black grains are an artifact of the preparation.

TABLE I

EFFECT OF SHEARING PRESSURE ON ERYTHROCYTE MEMBRANE ATPASE ACTIVITY

The numbers in parentheses refer to percent residual activity related to the respective activities of whole membranes. Shearing pressure refers to dial setting on hydraulic press.

Conditions	*ATPase activity (μmoles P_i liberated per h per mg protein)*									
Expt. No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Whole membranes										
Total ATPase	2.36	3.18	2.40	2.68	2.54	3.18	1.77	1.13	1.37	0.79
Mg^{2+}-activated ATPase	1.80	1.67	1.26	2.01	1.88	1.48	1.09	0.59	1.07	0.43
$(Na^{+}$-$K^{+})$-activated ATPase	0.56	1.51	1.14	0.67	0.66	1.70	0.68	0.54	0.30	0.36
Sheared membranes										
Shearing pressure 500 lb/inch^2										
Total ATPase	1.20 (51)		1.53 (64)	1.61 (60)	1.70 (67)	2.41 (76)	0.73 (41)	0.94 (83)	0.82 (60)	0.54 (68)
Mg^{2+}-activated ATPase	0.90 (50)		1.08 (85)	1.01 (50)	1.32 (70)	1.27 (86)	0.44 (40)	0.51 (86)	0.58 (54)	0.25 (58)
$(Na^{+}$-$K^{+})$-activated ATPase	0.30 (54)		0.45 (40)	0.60 (89)	0.38 (61)	1.14 (67)	0.29 (43)	0.43 (80)	0.24 (90)	0.29 (80)
Shearing pressure 1000 lb/inch^2										
Total ATPase		2.48 (78)								
Mg^{2+}-activated ATPase		1.50 (84)								
$(Na^{+}$-$K^{+})$-activated ATPase		0.98 (65)								
Shearing pressure 2000 lb/inch^2										
Total ATPase	0.79 (34)	1.72 (54)								
Mg^{2+}-activated ATPase	0.65 (35)	1.08 (65)								
$(Na^{+}$-$K^{+})$-activated ATPase	0.14 (25)	0.64 (42)								
Shearing pressure 5000 lb/inch^2										
Total ATPase	0.48 (20)	1.67 (52)								
Mg^{2+}-activated ATPase	0.42 (23)	0.94 (56)								
$(Na^{+}$-$K^{+})$-activated ATPase	0.06 (11)	0.73 (48)								

were performed by layering sheared membranes on 10–50 % linear sucrose gradients and centrifuging in the SW-25 rotor for 12–15 h. The membrane vesicles were resolved into two distinctly visible bands, one appearing one-third of the way from the bottom of the tube and the other two-thirds of the way up from the bottom. It was found that when the membrane vesicles were layered on a 30–45 % linear sucrose gradient and centrifuged for 5–6 h in the SW-39 rotor, an additional band could be partially separated just above the lowermost band. The protein content of the separated fraction was then measured as well as the ATPase activity. Because a maximum of 1.5 mg of membrane protein could be placed on each gradient, only microgram amounts were available for ATPase assay, which was therefore performed by the isotopic method. A pellet was not seen.

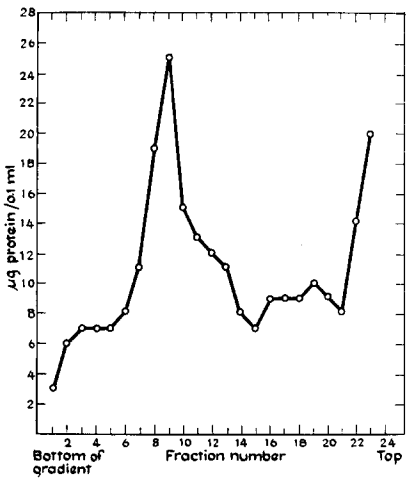

Fig. 2. Following centrifugation of the sucrose gradient, each tube was divided into approx. 20 fractions containing 3 drops each. Parallel fractions from the 3 tubes of the gradient were pooled. The protein content per 0.1 ml of sample is plotted against the fraction number. The peak of protein concentration occurring at Tubes 8 and 9 corresponds to the lowermost, visible dense band seen in the gradient, the material in Tubes 11–13 corresponds to a smaller band seen just above the lowermost band, while the material in Tubes 22 and 23 corresponds to the uppermost visible band.

The profile of protein content of the gradient separation is shown for one experiment in Fig. 2. ATPase assays were performed on samples taken from three portions of the gradient: the peak portion of the curve (Tubes 8 and 9), the extended shoulder (Tubes 11–13) and the top (Tubes 22 and 23). ATPase activities from these three segments are recorded for six experiments in Table III. In some experiments there was slight increment of specific activity. More regularly, the specific activity of (Na^+-K^+)-activated ATPase from the portion of the gradient containing the lowermost major peak was increased relative to the sheared membranes applied to the gradient. In contrast, the (Na^+-K^+)-activated ATPase was either reduced or undetectable at the top of the gradient where the specific activity of Mg^{2+}-activated ATPase was generally preserved.

The electron microscopic appearance of the sheared membranes applied to the gradient is identical to that in Fig. 1, and the electron microscopic appearance of the separated fractions is shown in Fig. 3. In all specimens the sheared membranes are

TABLE II

CENTRIFUGATION OF SHEARED MEMBRANES

Time of centrifugation at 175800 × g (min)	*Protein content*					*Total ATPase*					*Specific activity of total ATPase***	
	Supernatant		*Pellet*		*Total (mg)*	*Supernatant*		*Pellet*		*Total activity**	*Supernatant*	*Pellet*
	mg	%	*mg*	%		*Activity**	%	*Activity**	%			
0	3.54	100	0	—	3.54	2.05	100	0	—	2.05	0.58	0
5	2.66	76	0.83	24	3.49	1.32	70	0.56	30	1.88	0.49	0.67
13	1.84	55	1.50	45	3.34	0.72	37	1.23	63	1.95	0.42	0.82
33	1.15	37	1.95	63	3.10	0.36	18	1.69	82	2.05	0.31	0.87

* ATPase activity of the entire fraction expressed as μmoles P_i per h.
** Specific activity of ATPase expressed as μmoles P_i per h per mg protein.

TABLE III

ATPASE ACTIVITY OF DESIGNATED FRACTIONS COLLECTED FOLLOWING SUCROSE GRADIENT SEPARATION

Specific activity of ATPase expressed as μmoles P_i per h per mg protein. Numbers in parentheses indicate the percent of (Na^+-K^+)-activated ATPase relative to total ATPase.

Expt. No.	*ATPase of the material applied to the gradient*			*ATPase of the material collected from the major peak*			*ATPase of material collected from the shoulder*			*ATPase of material collected from the top*		
	Total	*Mg^{2+}-activated*	*(Na^+-K^+)-activated*	*Total*	*Mg^{2+}-activated*	*(Na^+-K^+)-activated*	*Total*	*Mg^{2+}-activated*	*(Na^+-K^+)-activated*	*Total*	*Mg^{2+}-activated*	*(Na^+-K^+)-activated*
1	0.28	0.15	0.13 (46)	0.25	0.11	0.14 (56)	0.21	0.16	0.05 (24)	0.14	0.14	0 (0)
2	0.18	0.14	0.04 (22)	0.18	0.12	0.06 (33)	0.14	0.09	0.05 (36)	0.09	0.09	0 (0)
3	0.32	0.21	0.11 (34)	0.33	0.21	0.12 (36)	0.27	0.17	0.10 (37)	0.17	0.15	0.02 (12)
4	0.19	0.10	0.09 (47)	0.53	0.21	0.32 (60)	0.30	0.15	0.15 (50)	0.38	0.36	0.02 (5)
5	0.34	0.16	0.18 (53)	0.66	0.39	0.27 (41)	0.51	0.35	0.16 (31)	—	—	—
6	0.54	0.44	0.10 (19)	0.57	0.38	0.19 (33)	0.50	0.30	0.20 (40)	0.12	0.11	0.01 (8)
Mean values	0.31	0.20	0.11 (36)	0.42	0.24	0.18 (43)	0.32	0.20	0.12 (38)	0.18	0.17	0.01 (6)

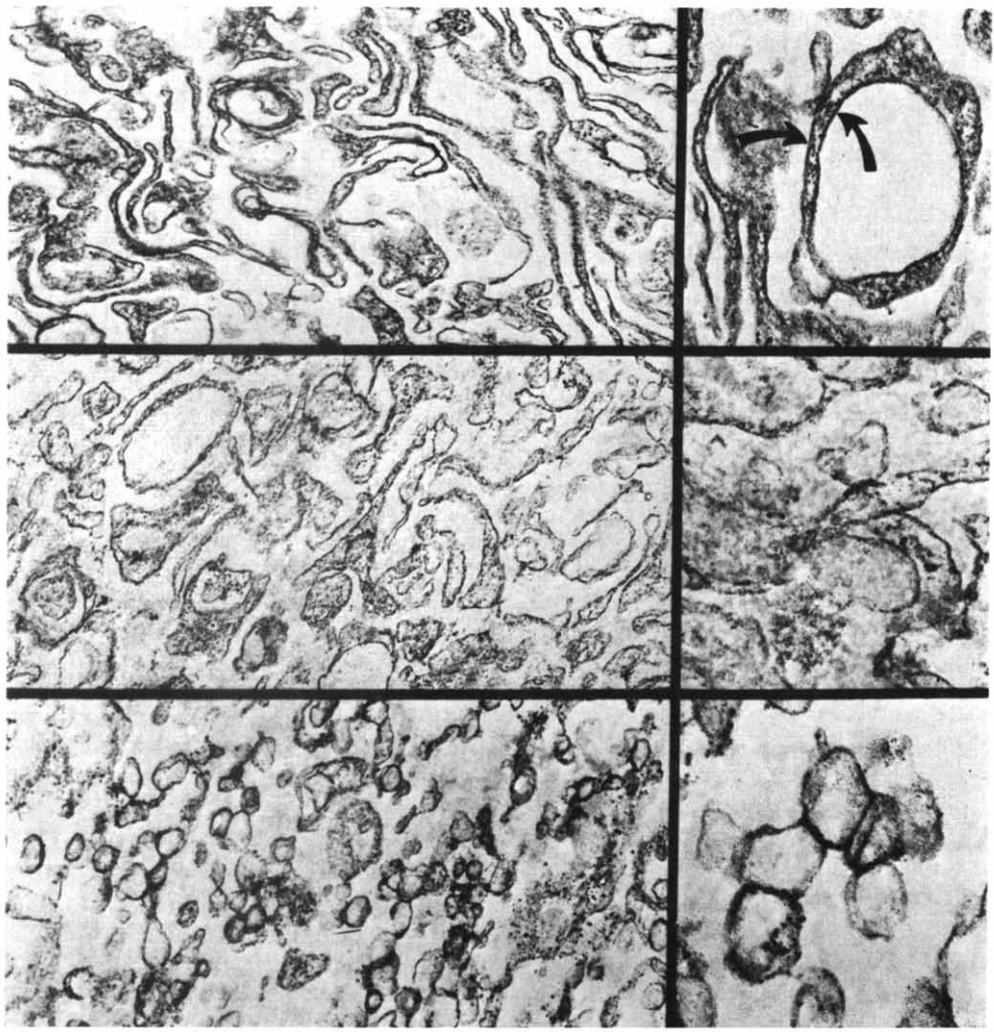

Fig. 3. Following density gradient centrifugation, membrane pellets prepared from the 3 major areas of the gradients were coded and examined. Photographs on the left are magnified 22 500 × while those on the right are magnified 45 000 ×. Top: membrane material from major peak of the gradient. Middle: membrane material from shoulder area of gradient. Bottom: membrane material from the top of the gradient. Arrows in top right indicate tri-lamellar unit membranes.

seen to consist of vesicles of varying size and form. The membrane material from the peak closest to the bottom of the gradient consists of the most dense particles containing the greatest proportion of recognizable tri-lamellar "unit membranes". These unit membranes are found on the "inside" and "outside" of the membrane vesicles. The membrane fragments from the "shoulder" portion of the gradient contain fewer examples of tri-lamellar structure and very few are recognized in the material at the top of the gradient.

The ability of oligomycin to completely inhibit (Na^+-K^+)-activated ATPase was used as a probe to determine other properties of the separated membrane

vesicles[10]. Membranes were sheared and separated by centrifugation in a 30–45 % linear sucrose gradient. In order to obtain the large amount of membrane material required, the fractions from each of the three tubes in the SW-39 rotor were pooled.

TABLE IV

OLIGOMYCIN INHIBITABILITY OF MEMBRANE (Na^+-K^+)-ACTIVATED ATPASE

Expts. 5 and 6 were performed using 30 mM imidazole–glycylglycine buffer instead of 20 mM Tris buffer, and the amount of added ATP was increased from 0.3 to 1.0 mM. In screening experiments we had determined that these changes resulted in increased specific activity of ATPase in the isotopic assay. Specific activity of ATPase expressed as μmoles P_i per h per mg protein. Percent oligomycin inhibition of (Na^+-K^+)-activated ATPase = 100 × (total ATPase − total ATPase with oligomycin)/(Na^+-K^+)-activated ATPase.

	ATPase of sheared membranes applied to gradient				
Expt. No.	*Total*	*Mg^{2+}-activated*	*(Na^+-K^+)-activated*	*Total + oligomycin*	*Oligomycin inhibition of (Na^+-K^+)-activated (%)*
1	0.21	0.17	0.04	0.16	> 100
2	0.18	0.14	0.04	0.12	> 100
3	0.19	0.15	0.04	0.15	100
4	0.54	0.44	0.10	0.44	100
5	1.27	0.93	0.34	0.93	100
6	1.21	1.10	0.11	1.09	100
	ATPase of membrane vesicles separated from major peak of the gradient				
Expt. No.	*Total*	*Mg^{2+}-activated*	*(Na^+-K^+)-activated*	*Total + oligomycin*	*Oligomycin Inhibition of (Na^+-K^+)-activated (%)*
1	0.24	0.17	0.07	0.18	86
2	0.18	0.12	0.06	0.16	33
3	0.23	0.12	0.11	0.17	55
4	0.57	0.38	0.19	0.49	42
5	1.82	1.16	0.66	1.64	27
6	1.57	1.01	0.56	1.15	75
				Mean	53
	ATPase of membrane vesicles separated from the shoulder of the gradient				
Expt. No.	*Total*	*Mg^{2+}-activated*	*(Na^+-K^+)-activated*	*Total + oligomycin*	*Oligomycin Inhibition of (Na^+-K^+)-activated (%)*
1	0.23	0.21	0.02	0.21	100
2	0.14	0.09	0.05	0.15	0
3	0.23	0.16	0.07	0.19	57
4	0.50	0.30	0.20	0.38	60
5	1.03	0.50	0.53	0.91	26
6	1.67	0.87	0.80	1.32	42
				Mean	48

The separated vesicles from the lowermost peak and from the shoulder of the gradient, as well as the sheared membranes applied to the gradient, were then subjected to study of their ATPase activities and the ability of oligomycin at a level of 60 μg/ml to inhibit (Na^+-K^+)-activated ATPase (Table IV).

While oligomycin completely inhibited (Na^+-K^+)-activated ATPase of unseparated, sheared membrane fragments suspended in 30% sucrose, it was only partially effective in inhibiting the (Na^+-K^+)-activated ATPase of the membrane vesicles obtained from the lower portions of the gradient where there was generally an increase in specific activity of (Na^+-K^+)-activated ATPase. This loss of sensitivity to inhibition by oligomycin was found in both segments of the gradient tested.

DISCUSSION

The microvesicles induced by shearing of membranes morphologically resemble those produced by ultrasonication of erythrocyte membranes[11] under special conditions consisting of hypotonic media and a gas–water interface. Under these conditions, the otherwise amorphous sonicate reaggregated into membranous films and vesicles, and it was further noted that addition of cations produced differing membranous films. Because it had been reported that Mg^{2+}-activated ATP protected ATPase against trypsin attack[12], we added Mg^{2+}, ATP, and Mg^{2+} ATP to membranes during the shearing process and discovered that 3 mM Mg^{2+} resulted in the most usable membrane vesicles for measurement of ATPase activity. It is probable that following high-pressure shearing, the dispersed membrane fragments reaggregate at air–water interfaces aided by the cation content of 3 mM Mg^{2+}. The resulting vesicles had diameters of approx. 0.6 μ and were sedimentable only when centrifugal forces in the order of 175000 $\times$ g were applied. Shearing of membranes resulted in loss of specific activity of ATPase in which both Mg^{2+}-activated ATPase and (Na^+-K^+)-activated ATPase shared equally at lower pressures. Shearing at higher pressures caused greater loss of ATPase activity, with no clear-cut preferential loss of either (Na^+-K^+)-activated ATPase or Mg^{2+}-activated ATPase.

When membrane vesicles resulting from shearing at a dial setting of 500 lb/inch^2 were placed on a 30–45% linear sucrose gradient and centrifuged at 175000 $\times$ g for 5 h, the vesicles were resolved into two or three visible bands. We cannot be absolutely sure whether buoyant density or particle size determined the position of the vesicles in the gradient. However, the size of vesicles examined from different portions of the gradient seemed to be approximately equivalent; therefore, we favor the formulation that relative buoyant density of the vesicle classes accounted for their position in the gradient. Those vesicles which retained most identifiable unit membrane structure and sedimented most rapidly contained the highest specific activity of total and (Na^+-K^+)-activated ATPase. Just above this band was another band which had less identifiable unit membrane structure visualized by electron microscopy and had generally good preservation of Mg^{2+}-activated ATPase and somewhat less (Na^+-K^+)-activated ATPase. In a band just under the meniscus there were also vesicles which contained least identifiable unit membranes and had lowest activity of (Na^+-K^+)-activated ATPase and total ATPase. This method, therefore, produces results which are substantially different from those previously described[13] where sonication of membranes in detergent yielded structureless material and where the membrane

material from the top part of the gradient contained the highest proportion of phospholipid and the highest (Na^+-K^+)-activated ATPase activity. Our method of preparation of the membrane microvesicles differed extensively from the conditions described by TOSTESON *et al.*[13] and these differences probably account for the lack of agreement in the two studies.

If pressure shearing of membranes resulted in the formation of a random spectrum of vesicle sizes and masses, the vesicles so formed would have been distributed diffusely and homogeneously throughout the sucrose gradient. However, two or three discrete bands of vesicles were formed either by the shearing process or during the reaggregation, and therefore, it is likely that the membranes were either cleaved in non-random fashion at repeating structurally weak loci or that they came together in relatively orderly manner. The observation that the different vesicle classes had different ATPase patterns and different ultrastructural characteristics bears out the suggestion of non-random cleavage and suggests that ATPases are localized in specific areas of a non-homogeneous membrane. The concept of heterogeneity in erythrocyte membranes has previously been raised by observation on the sites of hemoglobin extrusion during osmotic lysis[14] and by sites of cholesterol inclusion in the erythrocyte membranes[15].

There is a correlation between tri-lamellar membrane structure and increased specific activity of (Na^+-K^+)-activated ATPase and not Mg^{2+}-activated ATPase. This finding might indicate that preservation of a membrane structure is a requirement for (Na^+-K^+)-activated ATPase in erythrocyte membranes. Recent intriguing work on ATPases solubilized from brain microsomes by extraction with the detergent Lubrol indicates that the (Na^+-K^+)-activated ATPase is probably a lipoprotein[16,17]. If solubilization of (Na^+-K^+)-activated ATPase from human erythrocyte membranes can be achieved by similar techniques, it would suggest that the important consideration in our observations is the preservation of lipoprotein structure and not necessarily gross membrane structure.

Oligomycin completely inhibited the (Na^+-K^+)-activated ATPase of sheared but unseparated membrane vesicles, but only partly inhibited the (Na^+-K^+)-activated ATPase of separated membrane vesicles. There are several alternative explanations for this loss of oligomycin effect: structural alterations of the membrane vesicles might have diminished the accessibility of (Na^+-K^+)-activated ATPase to oligomycin, there might have been an increase in the ratio of phospholipid to protein in the vesicles, a change which has been reported to result in decreased oligomycin inhibition of the complex formed by β-hydroxybutyric dehydrogenase and micellar lecithin[18], or the shearing might have resulted in dislodgement of an oligomycin binding site. The small size of the vesicles tends to minimize, but not exclude considerations of accessibility of inhibitor to enzyme. If alteration in the ratio of phospholipid to protein was the controlling factor in oligomycin action, it is difficult to see why oligomycin was completely effective on the whole sheared preparation, but not on the separated vesicles. However, the loss of oligomycin inhibitability is consistent with the idea that resolution of the sheared vesicles on a sucrose gradient resulted in physical separation of a previously dislodged oligomycin combining site for the membrane vesicles. It is pertinent that an oligomycin sensitivity conferring protein has been described in mitochondria[19].

Attempts at recombination of material from different portions of the gradients

to restore susceptibility to oligomycin inhibition have been only partially successful thus far.

The use of sheared membrane vesicles provides the opportunity of studying membrane material which retains varying amounts of structure without the oxidative or interface problems encountered with ultrasonication.

The data obtained thus far indicates that, after shearing erythrocyte membranes into vesicles of varying size and structure, (Na^+-K^+)-activated ATPase is associated with fractions relatively rich in tri-lamellar membrane structures. The question about identity of Mg^{2+}-activated ATPase and (Na^+-K^+)-activated ATPase is not answered. It is likely that the binding site for oligomycin can be removed from the (Na^+-K^+)-activated ATPase without altering Na^+ and K^+ stimulation of the enzyme.

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