Separation of Adenosine Triphosphatase of HK and LK Sheep Red Cell Membranes by Density Gradient Centrifugation

D. C. TOSTESON, P. COOK, and R. BLOUNT

From the Department of Physiology and Pharmacology, Duke University, Durham

ABSTRACT Membrane fragments from high potassium (HK) and low potassium (LK) sheep red cells were separated by density gradient centrifugation. Three preparations were studied: (1) HK membranes sonicated for 20 minutes, (2) HK membranes sonicated for 3 minutes, and (3) LK membranes sonicated for 3 minutes. The adenosine triphosphatase (ATPase) activity in the maximally disrupted preparation (1) was not sensitive to Na + K and was recovered in relatively small but heavy (specific gravity 1.19) fragments which made up no more than 8 per cent of the total membrane. Both Na + K–sensitive (S) and Na + K–insensitive (I) ATPase activity were found in the more gently broken up preparations (2) and (3) but the ratio of S- to I-ATPase was much greater in HK than in LK membrane fragments. S-ATPase activity in preparation (2) was about 50 per cent that observed in HK membranes prior to sonication. S-ATPase activity was recovered from the density gradient in relatively large but light (specific gravity 1.10) fragments. As was the case with the maximally disrupted preparation (1), I-ATPase activity in both preparations (2) and (3) was recovered in small but heavy (specific gravity > 1.20) fragments. The possibility that sensitivity of sheep red cell membrane ATPase to Na + K depends on the association between units containing the enzyme(s) and large, light, phospholipid-containing components is discussed.

Considerable evidence suggests that a membrane adenosine triphosphatase (ATPase) activity which is stimulated by the simultaneous presence of sodium and potassium is involved in the active transport of Na out of, and K into, most animal cells (1–5). Both active transport of Na and K and Na + K–stimulated ATPase activity are about four times greater in high potassium (HK) than in low potassium (LK) sheep red cells (6, 7). Since the difference in red cell cation composition in the two types is determined by a chromosomal unit which segregates like a single gene (8), this system is particularly suitable for an attempt to define the molecular basis of active Na-K transport

1125

and, in particular, the role of Na + K–stimulated, membrane ATPase in the process.

Observations made with the electron microscope suggest that the structure of human (9) and sheep (14) red cell membranes is not homogeneous in its plane, but rather is composed of an array of plaques, each 200 to 300 A in diameter. The relationship between these plaques observed in air-dried, chromium-shadowed preparations and the structure of the membranes of intact erythrocytes is not yet well defined. Nevertheless, the possibility that the membrane is an aggregate of repeating units provided us with a working hypothesis for the isolation of membrane ATPase. If it is assumed that the average diameter of a plaque is 200 A, it may be estimated that there are about 300,000 such units per sheep red cell membrane. Kinetic and inhibitor studies (see Discussion) suggest that there are no more than 1000 to 3000 active transport (and Na + K–stimulated ATPase) sites per HK sheep red cell membrane and only about one-fourth that number per LK membrane. Therefore, if a site is no larger than one plaque, no more than 1 per cent of the plaques can possibly be directly involved in the process. This paper reports attempts to separate the active from the inactive units. Membranes were broken up into small pieces by exposure to ultrasound and detergent. The resultant fragments were separated by density gradient centrifugation and assayed for ATPase activity and other properties.

METHODS

Preparation of Membranes

Blood was drawn from the jugular vein of HK and LK sheep into heparin. The plasma and buffy coat were discarded and the red cells washed three times in 3 to 4 volumes of NaCl (0.17 M). All further steps in the preparation were carried out in the cold (0–5°C). The washed red cells were lysed in 10 volumes of tris(hydroxymethyl)-aminomethane (tris) chloride (0.07 M, pH 7.4). The lysate was placed in a large Buchner funnel over millipore filter paper (nominal pore diameter 0.8 micron) and stirred gently by a motor-driven paddle. Tris Cl (0.03 M, pH 7.4) was pumped into the funnel at a rate just equal to the rate at which membrane-free lysate flowed through the filter paper out of the funnel. Usually, 18 to 20 hours later the pale pink membranes were collected by centrifugation at 20,000 × g (Servall RC-2 refrigerated centrifuge with SS-34 rotor) and washed successively in 10 volumes of the following tris Cl solutions; once in 0.17 M, pH 7.4; twice in 0.04 M, pH 7.4; twice in 0.02 M, pH 7.4; twice in 0.01 M, pH 9.0, and finally once in 0.01 M, pH 7.4. An alternate procedure which produced equally good results was to complete the membrane preparation in the Buchner funnel by pumping first tris Cl (0.01 M, pH 9.0) and then tris Cl (0.01 M, pH 7.4) through the membrane suspension. The resultant membranes were white to the naked eye and had consistently good ATPase activity. All membrane suspensions were adjusted to contain 50 micromoles/ml of nitrogen before further processing.

Preparation of Membrane Fragments

1. HK 20 MINUTE SONICATE

15 ml of a suspension of HK membranes in tris Cl (0.01 M, pH 9.0) and polyoxy-ethylene-20-oleyl-ether (Brij 96) (0.5 per cent) were sonicated for 20 minutes with a Branson sonifier (20,000 cycles/second at a power output of *ca.* 65 watts). The sonication was carried out in a Rosett flask (10) in an ice bath which kept the temperature of the membrane suspension at 4°C. during the procedure. Metal particles which were generated from the probe of the sonifier were allowed to settle to the bottom of the Rosett flask before the membrane fragments were decanted.

2. HK 3 MINUTE SONICATE

15 ml of a suspension of HK membranes in tris Cl (0.01 M, pH 7.0) were sonicated for 3 minutes with a Branson sonifier. Other details of the sonication were identical with those noted above for the 20 minute HK preparation. Immediately after sonication, KCl (to make 0.17 M) and Brij 96 (to make 0.1 per cent) were added to the suspension of membrane fragments.

3. LK 3 MINUTE SONICATE

This preparation was identical with the HK 3 minute sonicate except that it was made from LK membranes.

The size of the membrane fragments in these preparations was estimated by filtering the suspensions through millipore filter paper of varying porosity. The amount of membrane material after each filtration was estimated by measuring the light (λ = 437 mμ) scattered at 90° with a Brice-Phoenix light-scattering photometer. Relative turbidities (as in Table I) are expressed as the ratio of light appearing at 90° to that at 0°.

Separation of Membrane Fragments by Density Gradient Centrifugation

1.0 ml aliquots of the suspension of membrane fragments were pipetted carefully on top of 4.0 ml of a linear 5 to 50 per cent sucrose gradient prepared in each of three 5 ml centrifuge tubes. In the case of preparation (1), the limiting solutions in the gradient also contained tris Cl (0.01 M, pH 9.0) and Brij 96 (0.5 per cent). In the case of preparations (2) and (3), the limiting solutions contained tris Cl (0.01 M, pH 7.0), Brij 96 (0.1 per cent), and KCl (0.17 M). Thus, each preparation of membrane fragments was placed on a sucrose gradient which differed from the solution in which the fragments were already suspended only in the presence of sucrose. The three tubes were placed in the swinging bucket rotor of a Spinco model L preparative ultracentrifuge and spun at 175,000 X *g*. Runs of differing durations showed that 48 hours was sufficient to bring most of the membrane fragments close to density equilibrium in the gradient. (See Results.) Characteristically, a visible band of membrane material was present in the tubes after centrifugation. The position of this band was measured by placing the tube in a ruled slot in a block of lucite.

6 samples were removed from the tubes when they were in this ruled slot. 4 holes were made with a hypodermic needle in the side of the tube at measured, roughly equally spaced intervals. The first hole was made about 1 cm below the level of the meniscus. Fluid flowing out of the hole was collected, without loss, in a Lang-Levy pipette. After collection of fraction 1 (top), a second hole 8 to 10 mm lower was made and fraction 2 taken, etc. Attempts to sample in the conventional manner through a hole in the bottom of the tube were not possible for two reasons. First, the visible band adhered to the walls of the tube to such an extent that it moved downward at a rate equal to the rate of fall of the meniscus only when its upper border became incorporated into the air-fluid interface. Thus, the conventional collection technique would have given the erroneous result that this material was located in the lightest, top fraction. Second, there was a visible pellet on the bottom of the tube which could contaminate samples drawn from the bottom of the tube. This pellet was resuspended in tris Cl (0.01 M, pH 9.0 for preparation (1) and 0.01 M, pH 7.0 for preparations (2) and (3)), after removal of the bottom layer of fluid (fraction 5) from above with a Lang-Levy pipette. Fractions from each of the three tubes were pooled and the volume measured in a graduated tube.

Analyses of Fractions

SPECIFIC GRAVITY

The specific gravities of the limiting solutions were measured by weighing equal volumes of solution and water at 24.7°C. Osmolalities of limiting solutions as well as of fractions taken from the gradient were estimated from the freezing point depression measured with a Fiske osmometer. Specific gravities of fractions were then computed by interpolation. Comparison of this method with direct measurement (by weighing) of specific gravities of mixtures of the limiting solutions showed that the two procedures gave identical results. The greater speed, convenience, and volume economy of the osmometric-interpolation method led to its routine use.

OPTICAL DENSITY

The optical densities of starting material and all fractions at wave lengths of 260 and 280 mm were measured with a Zeiss PM Q II spectrophotometer.

NITROGEN

The nitrogen content of starting material and all fractions was measured by the microKjehldahl procedure.

PHOSPHORUS

Total phosphorus in starting material and in all fractions was determined by the method of Bartlett (11). Lipid phosphorus was determined in some of the fractions as suggested by Marinetti (12). Lipid was extracted from 1 volume of packed membranes with 50 volumes of chloroform-methanol (1:1). The extract was evaporated to dryness and analyzed for total P as above (11). In both procedures, it was usually necessary to add hydrogen peroxide several times to produce clearing after digestion.

Standard phosphate samples received the same amount of H_2O_2 to control the amount of contaminating phosphorus present in this reagent.

ATP

Welt *et al.* (13) have recently shown that both HK and LK sheep red cell membranes contain a small and equal amount of bound ATP. Bound ATP was released from the membrane fragments in starting material and all fractions by boiling. This method gave the same results as exposure to perchloric acid (13). Released ATP was measured with the luciferin-luciferase system obtained from firefly tails (Worthington). The light emitted from the reaction was detected by a scintillation spectrometer (Packard). Known amounts of ATP added to the firefly system were used as standards. Prior to boiling, the membrane fragments contained no ATP detectable by this method (less than 0.2×10^{-12} M/10^{-6} M nitrogen).

ATPASE

Starting material and all fractions were assayed for ATPase activity under three different conditions. In all three conditions, the reaction mixture had a volume of 2.5 ml and contained ATP (2×10^{-6} M), $MgCl_2$ (10^{-4} M), tris Cl (0.017 M, pH 7.4), and membrane fragments (0.2 ml, 0.5–10×10^{-6} M nitrogen depending on fraction assayed). In the first condition (K only), the mixture also contained KCl (0.1 M). In the second condition (Na + K) the mixture also contained NaCl (0.08 M) and KCl (0.02 M). In the third condition (Na + K + St), the mixture also contained NaCl (0.08 M), KCl (0.02 M), and strophanthidin (St) (10^{-4} M). Activities in the first and third conditions were always about the same and the mean of these values was taken as insensitive ATPase (I-ATPase). Sensitive ATPase (S-ATPase) was calculated as the difference between activity in Na + K and insensitive ATPase. The reaction was started by addition of ATP after 30 minutes of preincubation at 37°C of all other components. The reaction was stopped after 15 to 100 minutes, at 37°C (depending on the amount of activity in the fraction under assay) by placing the reaction tubes in a boiling water bath for 1 minute. This procedure was proven to completely inactivate this membrane ATPase. The amount of ATP remaining in the reaction flasks was measured by the luciferin-luciferase system as described above. Known amounts of ATP added to reaction flasks of composition identical to those used in the assay except that the ATPase activity was inactivated by boiling, were used as standards. The amount of bound ATP released on boiling was negligible compared to the amount of ATP added in carrying out the reaction. In general, the ATPase reaction was stopped when no more than half of the ATP present had been hydrolyzed. Because of its greater sensitivity, this method allowed assay of ATPase activity on smaller amounts of membrane fragments and in shorter times than the more conventional procedure of measuring release of inorganic phosphate.

RESULTS

Size of Membrane Fragments

The effect of exposure of HK and LK membranes suspended in tris Cl (0.01 M, pH 7.0) to ultrasound for 3 minutes is shown in Fig. 1. Note the
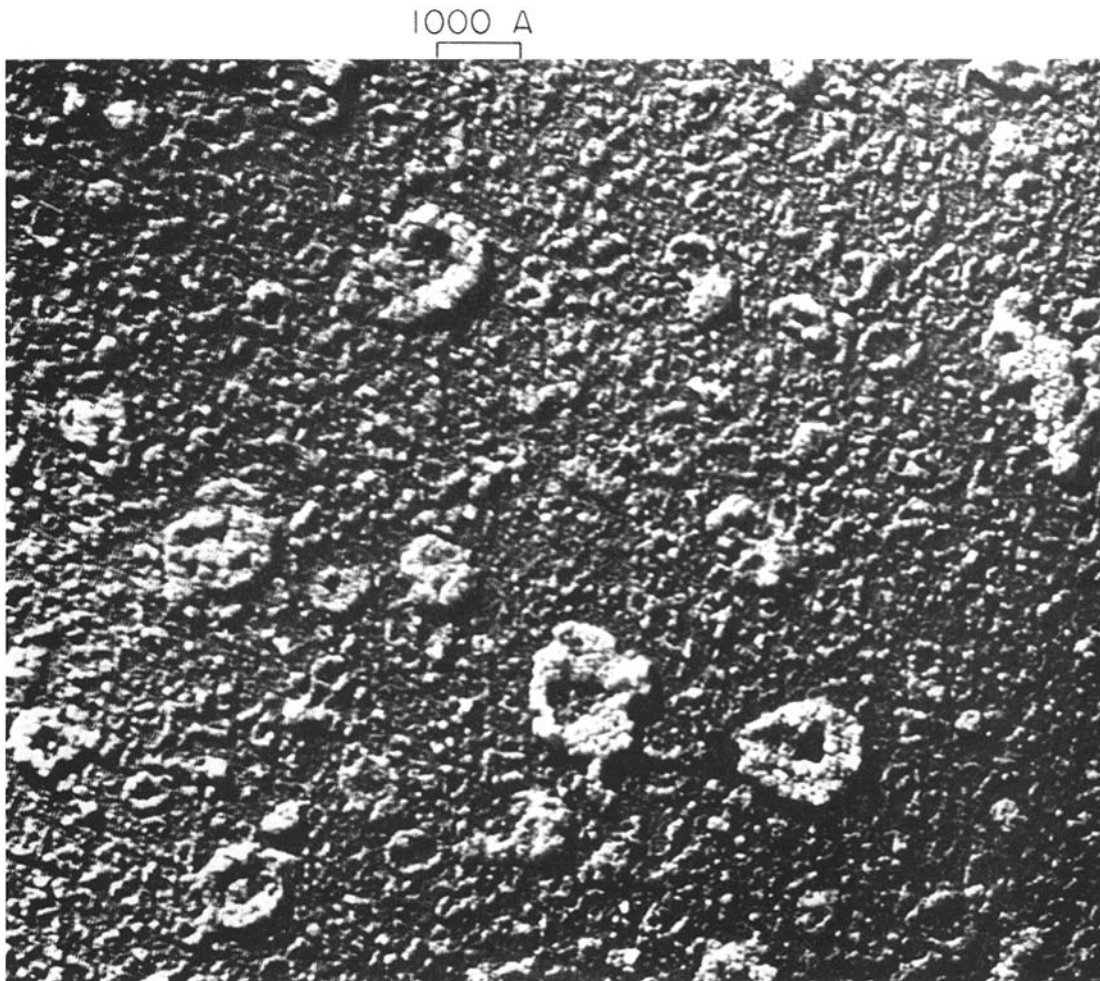
FIGURE 1.   This is an electron micrograph of HK sheep red cell membrane fragments. The fragments were prepared by sonicating membranes (rendered hemoglobin-free by successive osmotic lysis, see text) for 3 minutes. A drop of suspension of fragments was air-dried on a collodion grid and chromium-shadowed (angle 4:1) prior to electron microscopy. × 110,000.

presence of small units 200 to 300 A in diameter alone and in various states of aggregation. Some aggregates are as large as 2000 A in diameter. It is not possible to distinguish between aggregates formed by recombination of units which were separated at the time of sonication and pieces of the membrane which were not broken up by ultrasound.

An estimate of the size of membrane fragments in the three preparations

studied is shown in Table I. Note that virtually all the membrane material in the 3 minute sonicates is removed by passage through filter paper with 1000 A pores whereas a substantial fraction (*ca.* 20 per cent) of the material in the 20 minute sonicate passes through this filter. It is not strictly valid to use turbidity as a measure of the amount of membrane material since the magnitude of light scattering by the fragments depends on their size. Thus, this method overestimates the amount of large material and underestimates the amount of small material. This point is emphasized by the fact that all

TABLE I

FILTRATION SIZING OF MEMBRANE FRAGMENTS

This table shows the effect of filtration of a suspension of membrane fragments through Millipore filter paper with different nominal pore diameters. Each row shows properties of the suspension after passage through the specified filter. Relative turbidity is proportional to the ratio of light scattered at 90° to that passing directly through the suspension. *D* is the dissymmetry ratio; *i.e.*, the ratio of light scattered at 45° to that scattered at 135° from the incident beam. Values in the column headed per cent total membrane were computed from the ratio of relative turbidity after filtration through a given filter to the relative turbidity of the unfiltered suspension. For further details, see text.

Nominal diameter of filter pores	HK 20 min. sonicate Experiment 192			HK 3 min. sonicate Experiment 190			LK 3 min. sonicate Experiment 194		
	Relative turbidity 90/0	D 45/135	Total membrane	Relative turbidity	D 45/135	Total membrane	Relative turbidity 90/0	D 45/135	Total membrane
	per cent				*per cent*				*per cent*
Unfiltered	0.152	4.18	100	1.02	3.88	100	0.833	3.66	100
4500 A	0.095	2.53	63	0.775	3.21	78	0.720	3.22	86
2200 A	0.065	1.44	43	0.487	2.74	49	0.462	2.83	55
1000 A	0.030	1.32	20	0.049	2.19	5	0.005	2.15	1
500 A	0.021	1.46	14	0.000	0.99	0	0.000	1.52	0
100 A	0.008	1.64	5	0.000	0.99	0	0.000	0.92	0

three preparations contained the same concentration of membrane material yet the turbidity of the 20 minute sonicate was only 15 to 20 per cent that of the 3 minute sonicates. An independent measure of the relative size of the fragments in the different preparations is given by the dissymmetry ratio (*D*), the ratio of light scattered at 45° to that scattered at 135° to the incident beam. Note that *D* decreases progressively with filtration in all three preparations.

It is important to note that the conditions of fragmentation in the 20 minute and 3 minute preparations differed in more respects than simply in the duration of sonication. Ionic strength, specific metal ions, pH, and concentration and molecular properties of detergent are all important factors in determining

separation and aggregation of membrane units. A report on the relative roles of these factors in producing aggregation of membrane units is in preparation (14). The two methods of preparation of membrane fragments used in these experiments were selected because one (20 minute sonicate in Brij 96 (0.5 per cent) and tris Cl (0.01 M, pH 9.0)) gave maximum fragmentation at the expense of loss of sensitivity of ATPase activity to Na + K, while the other (3 minute sonicate in tris Cl (0.01 M, pH 7.0) with subsequent addition of KCl (0.17 M) and Brij 96 (0.1 per cent)) gave the greatest fragmentation consistent with retention of sensitivity of ATPase to Na + K (Table II).

TABLE II

SHEEP RED CELL MEMBRANE ATPase

EFFECT OF PREPARATION CONDITIONS ON
ACTIVATION BY Na + K

This table shows the effect of duration and conditions
of sonication of HK sheep red cell membrane ATPase.
Note loss of Na + K–sensitive activity after sonication
for 20 minutes but partial retention of this activity after
sonication for 3 minutes.

| Preparation | ATPase 10^{-12} M | | |
| | (min.) $\times$ (10^{-4} M nitrogen) | | |
	K	Na + K	Na + K + strophanthidin
Intact membranes	6.9	17	7.0
20 min. sonicate 0.5 per cent Brij 96	11	10	11
3 min. sonicate 0.1 per cent Brij 96 0.17 M KCl	5.7	11	5.6
Experiment 165			

Separation of Fragments Produced by Sonication of HK Sheep Red Cell Membranes for 20 Minutes

The results of separation of the HK 20 minute sonicate by density gradient centrifugation are shown in Table III. A visible band about 1 mm thick was noted in fraction 4, 3.0–3.4 cm from the bottom of the tube. Note that in this greatly broken up preparation the specific gravity of the visible band (1.05) was less than the major fraction of membrane nitrogen (1.07) but greater than the major fraction of membrane phosphorus (1.02). Furthermore, most of the membrane-bound ATP was found in fragments with a still greater density (1.14). This separation of visible band, membrane nitrogen, phosphorus, and ATP from one another did not occur in the more gently broken up HK and LK membranes which were sonicated for only 3 minutes

TABLE III

HK MEMBRANE FRAGMENTS PREPARED
BY SONICATION FOR 20 MINUTES

Hemoglobin-free HK sheep red cell membranes prepared by successive osmotic lysis were suspended in 0.01 M tris Cl (pH 9.0) containing 0.5 per cent Brij 96 and sonicated for 20 minutes. 3.0 ml of the resultant suspension of membrane fragments were layered on top of a linear 5 to 50 per cent sucrose gradient. After centrifugation, fractions were collected by puncturing the side of the tube and analyzed. Insensitive ATPase is defined as the mean of activity in the presence of K only and Na + K + strophanthidin. Sensitive ATPase is taken as the difference between activity in Na + K and insensitive ATPase. Negative values for sensitive ATPase occur when activity is actually less in Na + K than in K alone or Na + K + strophanthidin. Peak values for each material analyzed are set in bold-face figures. ATPase activities are expressed in two ways, as specific activity (10^{-12} M/(min.) $\times$ (10^{-6} M nitrogen)) and as per cent of the total activity placed on the gradient. The latter value was computed from the ratio of ATPase activity in a given fraction to the activity placed on the gradient. "Per cent total" figures in the row designated "sum of fractions" indicate recovery of material placed on gradient. Specific activity figures in this row were computed by dividing total recovered enzyme activity by total recovered nitrogen. The experiment shown in this table is representative of five, one each at centrifugation time of 12, 24, 36, 48, and 60 hours.

			Centrifugation. 175,000 g, 48 hrs.						Experiment 193					
			Nitrogen		Phosphorus		Bound ATP		ATPase					
									Na + K–insensitive		Na + K–sensitive		Ratio
									Specific activity 10^{-12} M		Specific activity 10^{-12} M		
Fraction	Distance From bottom of tube	Specific gravity 25°C	10^{-6} M	Total	10^{-6} M	Total	10^{-9} M	Total	(Min.) $\times$ (10^{-6} M nitrogen)	Total	(Min.) $\times$ (10^{-6} M nitrogen)	Total	S/I
	cm			per cent		per cent		per cent		per cent		per cent	
Starting material	—		135	100	9.21	100	7.42	100	5.76	100	−0.20	—	0.0
Pellet	0.0	>1.19	7.8	6	0.09	1	0.02	0	31.3	31	−3.20	—	−0.1
Fraction 1	0.0–1.1	1.19	10.2	8	0.13	1	0.83	11	58.3	**76**	−1.90	—	0.0
Fraction 2	1.1–2.2	1.14	35	26	0.24	3	4.72	**64**	2.70	12	+0.40	—	−0.1
Fraction 3	2.2–3.0	1.07	56	**42**	0.72	8	0.92	12	0.01	1	−0.01	—	0.0
Fraction 4 (band)	3.0–3.4	1.05	12	9	2.37	26	0.04	1	5.28	8	−0.14	—	0.0
Fraction 5	3.4–4.3	1.02	8.4	6	4.55	**49**	0.02	0	10.4	11	+0.55	—	0.0
Sum of fractions			129	97	8.10	88	6.55	88	8.44	139	−0.27	—	0.0

(see below). All the membrane ATPase activity placed on and recovered from the sucrose density gradient was insensitive to Na + K. There was no S-ATPase activity in this preparation. Most of the I-ATPase activity was

TABLE IV

HK MEMBRANE FRAGMENTS PREPARED
BY SONICATION FOR 3 MINUTES

Hemoglobin-free HK sheep red cell membranes prepared by successive osmotic lysis were suspended in 0.01 M, tris Cl (pH 7.0) and sonicated for 3 minutes. The resultant suspension of membrane fragments was brought to 0.17 M KCl and 0.1 per cent Brij 96 by addition of these compounds. 3.0 ml of this suspension were layered on top of a linear 5 to 50 per cent sucrose gradient. After centrifugation fractions were collected by puncturing the side of the tube and analyzed. Insensitive ATPase is defined as the mean activity in the presence of K only and in Na + K + strophanthidin. Sensitive ATPase is taken as the difference between activity in Na + K and insensitive ATPase. Negative values for sensitive ATPase occur when activity is actually less in Na + K than in K alone or Na + K + strophanthidin. Peak values for each material analyzed are set in bold-face figures. ATPase activities are expressed in two ways, as specific activity (10^{-12} M/(min.) $\times$ (10^{-6} M nitrogen)) and as per cent of the total activity placed on the gradient. The latter value was computed from the ratio of ATPase activity in a given fraction to the activity placed on the gradient. Per cent total figures in the row designated sum of fractions indicate recovery of material placed on gradient. Specific activity figures in this row were computed by dividing total recovered enzyme activity by total recovered nitrogen. The experiment shown in this table is representative of four, with centrifugation times of 16 (1 experiment), 36 (1 experiment), and 48 (2 experiments) hours.

			Centrifugation: 175,000 g, 48 hrs.						Experiment 197				
Fraction	Distance from bottom of tube	Specific gravity 25°C	Nitrogen		Phosphorus		Bound ATP		ATPase				
									Na + K–insensitive		Na + K–sensitive		Ratio
									Specific activity 10^{-12} M		Specific activity 10^{-12} M		
									(Min.) × (10^{-6} M nitrogen)	Total	(Min.) × (10^{-6} M nitrogen)	Total	S/I
			10^{-8} M	Total	10^{-6} M	Total	10^{-9} M	Total					
	cm			per cent		per cent		per cent		per cent		per cent	
Starting material			141	100	9.45	100	—		7.16	100	4.66	100	0.6
Pellet	0.0	>1.20	8.4	6	0.06	1	0.04	1	55.0	**46**	−0.71	−1	0.0
Fraction 1	0.0–1.1	1.20	19	13	0.46	5	0.16	3	13.7	26	1.63	5	0.1
Fraction 2	1.1–2.0	1.16	27	19	2.15	23	0.54	11	9.27	25	0.11	1	0.0
Fraction 3 (band)	2.0–2.6	1.10	51	**36**	4.14	**44**	2.40	**49**	1.72	9	11.4	**87**	**6.6**
Fraction 4	2.6–3.3	1.06	34	24	3.92	41	1.82	37	4.31	14	9.62	48	2.2
Fraction 5	3.3–4.2	1.03	0.0	0	0.49	5	—	—	—	17	—	1	0.0
Sum of fractions			139	98	11.22	119	4.96	100	9.94	137	6.75	141	0.7

TABLE V

LK MEMBRANE FRAGMENTS PREPARED
BY SONICATION FOR 3 MINUTES

Hemoglobin-free LK sheep red cell membranes prepared by successive osmotic lysis were suspended in 0.01 M tris Cl (pH 7.0) and sonicated for 3 minutes. The resultant suspension was brought to 0.17 M KCl and 0.1 per cent Brij 96 by addition of these compounds. 3.0 ml of the resultant suspension of membrane fragments were layered on top of a linear 5 to 50 per cent sucrose gradient. After centrifugation, fractions were collected by puncturing the side of the tube and analyzed. Insensitive ATPase is defined as the mean of activity in the presence of K only and Na + K + strophanthidin. Sensitive ATPase is taken as the difference between activity in Na + K and insensitive ATPase. Negative values for sensitive ATPase occur when activity is actually less in Na + K than in K alone or Na + K + strophanthidin. Peak values for each material analyzed are set in bold-face figures. ATPase activities are expressed in two ways, as specific activity $(10^{-12}$ M/(min.$) \times (10^{-6}$ M nitrogen$))$ and as per cent of the total activity placed on the gradient. The latter value was computed from the ratio of ATPase activity in a given fraction to the activity placed on the gradient. Per cent total figures in the row designated sum of fractions indicate recovery of material placed on gradient. Specific activity figures in this row were computed by dividing total recovered enzyme activity by total recovered nitrogen. The experiment shown in this table is representative of three, two centrifuged for 48 and one for 36 hours.

			Centrifugation time: 175,000 g, 48 hrs.						Experiment 198				
			Nitrogen		Phosphorus		Bound ATP		ATPase				
									Na + K–insensitive		Na + K–sensitive		Ratio
Fraction	Dis-tance from bottom of tube	Specific gravity 25°C	10^{-6} M	Total	10^{-4} M	Total	10^{-9} M	Total	Specific activity 10^{-12} M (Min.) $\times$ $(10^{-6}$ M nitrogen)	Total	Specific activity 10^{-12} M (Min.) $\times$ $(10^{-6}$ M nitrogen)	Total	S/I
	cm			per cent		per cent		per cent		per cent		per cent	
Starting material			153	100	9.90	100	—	—	4.16	100	−0.37	—	−0.1
Pellet	0.0	>1.20	20	13	0.10	1	0.03	0	18.7	57	−0.46	−11	0.0
Fraction 1	0.0–1.1	1.20	14	9	0.60	6	0.32	5	20.4	46	0.00	0	0.0
Fraction 2	1.1–2.0	1.15	24	16	0.55	5	0.60	9	7.16	27	−1.45	−42	−0.2
Fraction 3 (band)	2.0–2.6	1.10	57	37	4.20	42	3.15	48	0.54	5	1.20	82	2.2
Fraction 4	2.6–3.3	1.06	31	20	5.40	54	2.48	38	1.25	6	1.45	54	1.2
	3.3–4.2	1.03	6.6	5	0.55	5	0.03	0	6.25	6	2.12	17	0.3
Sum of fractions			153	100	11.30	113	6.61	100	6.18	147	0.55	100	0.1

present in fragments which had a specific gravity of 1.19 and contained about 8 per cent of the total membrane nitrogen. Thus, this relatively heavy I-ATPase was purified about tenfold by density gradient centrifugation.

Separation of Fragments Produced by Sonication of HK Sheep Red Cell Membranes for 3 Minutes

The equilibrium distribution in the density gradient of membrane fragments produced by this more gentle procedure is shown in Table IV. The visible

TABLE VI

EFFECT OF DURATION OF CENTRIFUGATION
ON Na + K-INSENSITIVE ATPase

Both experiments in each pair were carried out with fragments prepared from the same batch of membranes. The specific gravities of the fractions of the HK 20 minute sonicate were lighter than those of HK and LK 3 minute sonicates. Therefore, the enzyme activities for these two types of preparation are listed separately (HK 20 min. on left, HK and LK 3 minute on right of each column) under the corresponding specific gravity. For details see text.

Membrane fragment preparation	Duration of centrifugation at force of 175,000 g	Total activity recovered in several fractions of different specific gravities										
		>1.20	1.19	1.20	1.14	1.16	1.07	1.10	1.04	1.06	1.02	1.03
		per cent	*per cent*		*per cent*		*per cent*		*per cent*		*per cent*	
HK	36 hrs. (Experiment 192)	8	72		8		0		5		8	
20 min. sonicate	48 hrs. (Experiment 193)	22	54		9		1		6		8	
HK	36 hrs. (Experiment 190)	9		32		19		15		12		14
3 min. sonicate	48 hrs. (Experiment 191)	22		21		22		6		13		16
LK	36 hrs. (Experiment 194)	13		29		29		12		8		9
3 min. sonicate	48 hrs. (Experiment 195)	20		24		17		15		10		14

band was broader (3 to 4 mm), lower in the centrifuge tube (2.0 to 2.6 cm from the bottom), and heavier (specific gravity 1.10) in this preparation than in the 20 minute sonicate. Furthermore, the peak amounts of membrane nitrogen, phosphorus, and ATP were all found in fragments with the specific gravity (1.10) of this visible band. Thus, the more gentle treatment of the membranes produced fragments in which most of the membrane nitrogen, phosphorus, and ATP remained associated with one another. The ratio of Na + K-sensitive to Na + K-insensitive ATPase activity in the starting

material was 0.6. Most of the I-ATPase activity was recovered from the density gradient in relatively heavy fragments (specific gravity >1.20). In contrast, virtually all the S-ATPase activity was recovered from the density gradient in relatively light fragments (specific gravity 1.10), the same fragments which contained the bulk of membrane nitrogen, phosphorus, and ATP. The ratio of Na + K–sensitive to Na + K–insensitive activity of 6.6 indicates that the ATPase activity in these relatively light fragments was almost completely dependent on Na + K.

TABLE VII

EFFECT OF DURATION OF CENTRIFUGATION ON
DISTRIBUTION OF Na + K–SENSITIVE ATPase

HK Membrane Fragments Prepared by Sonication for 3 Minutes

Experiments 190 and 191 were done with one while 196 and 197 were
done with another batch of HK membranes. For details see text.

Duration of centrifugation at force of 175,000 g	Total activity recovered in several fractions of different specific gravities					
	per cent >1.19	*per cent* 1.19	*per cent* 1.16	*per cent* 1.11	*per cent* 1.06	*per cent* 1.03
36 hrs. (Experiment 190)	2	−2	4	72	22	2
48 hrs. (Experiment 191)	−1	4	1	78	19	−2
16 hrs. (Experiment 196)	1	−2	−2	68	35	0
48 hrs. (Experiment 197)	−1	3	1	62	35	0

Separation of Fragments Produced by Sonication of LK Sheep Red Cell Membranes for 3 Minutes

As was the case with the HK 3 minute preparation, visible band, membrane nitrogen, and ATP in the LK preparation were all concentrated in the same, relatively light (specific gravity 1.10) fraction (Table V). All the ATPase activity in this particular sonicate (prior to centrifugation) was insensitive to Na + K. Most of this I-ATPase activity was recovered in a relatively heavy fraction (specific gravity > 1.20). Thus, the I-ATPase activity in all three preparations is relatively heavy. A small but consistent amount of Na + K–sensitive ATPase activity was observed in the specific gravity 1.10 fragments of these LK membranes. This S-ATPase activity was completely masked by I-ATPase activity in this particular LK sonicate prior to separation in the density gradient.

Some Effects of Duration of Centrifugation

The effect of duration of centrifugation on the distribution of I-ATPase activity in the density gradient is shown in Table VI. In all three preparations studied, there is a definite increase in the amount of activity in the pellet (specific gravity > 1.19) when the centrifugation time is increased from 36 to 48 hours. There is a corresponding decrease in the activity found in the next lighter fraction. These data indicate that much of the I-ATPase in HK and LK sheep red cell membranes is located in relatively small but heavy fragments. Accurate estimation of the density of these fragments must await further work with the heavier density gradients.

TABLE VIII

EFFECT OF DURATION OF CENTRIFUGATION ON
SENSITIVITY OF ATPase TO Na + K HK MEMBRANE FRAGMENTS
PREPARED BY SONICATION FOR 3 MINUTES

Both experiments in each pair (Experiments 190-191 and 196-197) were
done with the same batch of membranes. For details, see text.

Preparation	Duration of centrifugation at force of 175,000 g	Specific activity of ATPase in 10^{-12} M/(min.) × (10^{-4} M nitrogen)		
		Na + K insensitive	Na + K sensitive	Ratio S/I
Starting material	0 hrs. (Experiments 190, 191)	2.97	5.12	1.7
Specific gravity Fraction 1.10	36 hrs. (Experiment 190)	5.04	9.74	1.9
	48 hrs. (Experiment 191)	1.42	17.5	12
Starting material	0 hrs. (Experiments 196, 197)	7.87	3.42	0.4
Specific gravity Fraction 1.10	16 hrs. (Experiment 196)	3.62	10.0	2.8
	48 hrs. (Experiment 197)	1.72	11.4	6.6

The effect of duration of centrifugation on the distribution of S-ATPase is shown in Table VII. Since the 3 minute HK sonicate was the only preparation which contained substantial amounts of this activity, the table presents data obtained with this system. Note that the distribution of S-ATPase in the density gradient was at equilibrium after no more than 16 hours of centrifugation. Likewise, the distribution of the visible band, membrane nitrogen, phosphorus, and ATP remained unchanged after 16 hours. These results suggest that S-ATPase is located in relatively large, light fragments which rapidly come to density equilibrium in the centrifuge.

Another interesting effect of duration of centrifugation is shown in Table VIII. These data show that the ratio of Na + K–sensitive to Na + K–insensitive ATPase activity in the specific gravity 1.10 fraction increases with in-

creasing duration of centrifugation. This effect is due partly to an increase in specific activity of the S-ATPase but mainly to a decrease in I-ATPase.

The Nature of Membrane Phosphorus

Tables III, IV, and V show that most of the membrane phosphorus is recovered from the gradient in the relatively light fractions. The P/N ratio is greatest in these fragments. Table IX shows that virtually all the phosphorus found in the specific gravity 1.10 fraction (which contains Na + K–sensitive ATPase) is lipid phosphorus.

TABLE IX

LIPID AND TOTAL P IN MEMBRANE FRAGMENTS

3 minute sonicate

This table shows the amount of total and lipid phosphorus found in the specific gravity 1.10 fraction of 3.0 ml of packed membranes. This fraction contains most of the S-ATPase.

	Experiment 196		Experiment 195	
	HK		LK	
Membrane preparation	Total P	Lipid P	Total P	Lipid P
	10^{-4} M			
Specific gravity Fraction 1.10	4.60	4.16	4.62	3.84

Effect of Separation of Membrane Components on Sensitivity of ATPase to Na + K

Because the above data suggested that Na + K sensitivity of the membrane ATPase depends on the association between the enzyme(s) and light, phospholipid-containing components, several experiments were carried out to examine this possibility. First, extensive disruption of HK membranes by exposure to ultrasound for 20 minutes in the presence of tris Cl (0.01 M, pH 9.0) and Brij 96 (0.5 per cent) produced loss of Na + K sensitivity but not loss of total ATPase activity. That is, loss of Na + K–sensitive ATPase resulted from an increase in Na + K insensitive–ATPase rather than a decrease in total ATPase activity. This point is shown in Table X. Note that virtually all the membrane ATPase was recovered in the heavy pellet which in this experiment contained only 6 per cent of the membrane nitrogen. Second, mere separation of the membrane fragments in the density gradient usually produced loss of Na + K–sensitive ATPase and gain of Na + K–insensitive ATPase with little change in total membrane ATPase activity. Typical results of this kind obtained with HK 3 minute sonicates are

TABLE X

EFFECT OF SONICATION FOR 20 MINUTES ON
HK SHEEP RED CELL MEMBRANE ATPase

This table shows the recovery of ATPase activity present
in HK membranes after sonication for 20 minutes in
tris Cl (0.01M, pH 9.0) and Brij 96 (0.5 per cent). The
activities in the pellet and the sum of all fractions ob-
tained from a density gradient centrifugation separation
of the sonicate are also shown. For interpretation, see
text.

Preparation	Relative ATPase activity			
Experiment 103	Total	Na + K sensitive	Na + K insensitive	S/I
Intact membranes	100	84	16	5.2
Sonicate	77	0	77	0.0
Pellet fraction	79	0	79	0.0
Sum of gradient fractions	79	0	79	0.0

shown in Table XI. Not all experiments yielded such findings. Occasionally,
particularly in the case of sonicates which had relatively little Na + K–sen-
sitive ATPase (e.g., the experiment shown in Table IV), "uncoupling" due
to separation alone was not evident. The loss of Na + K sensitivity usually
observed during separation in the ultracentrifuge was clearly not due to the
effect of sucrose because certain gradient fractions contained much Na +
K–sensitive ATPase (see Table IV).

TABLE XI

EFFECT OF SEPARATION ON Na + K SENSITIVITY
OF 3 MINUTE HK SONICATE

This table shows the recovery of ATPase activity in a
3 minute sonicate of HK membranes after separation by
density gradient centrifugation. The activity found in
the sum of all the gradient fractions is compared with
that placed on the gradient. For interpretation, see text.

Preparation	Relative ATPase			
	Total	Na + K sensitive	Na + K insensitive	S/I
Experiment 175 sonicate	100	58	42	1.37
Experiment 175 sum of gradient fractions	109	37	72	0.52
Experiment 149 sonicate	100	86	14	6.1
Experiment 149 sum of gradient fractions	98	37	61	0.6

Effect of Recombination of Membrane Components

Thus, the observations noted above favor the view that Na + K sensitivity depends on the relation of the catalytic system to adjacent membrane units. If this conclusion is correct, it should be possible to recombine the separated light, Na + K sensitivity–conferring units with the heavy I-ATPase units to produce an Na + K–sensitive ATPase complex. We have made a number of attempts to carry out this experiment without definitive success. These experiments were done by assaying the Na + K–sensitive and insensitive ATPase activity in heavy and light fractions before and after recombination. Comparisons were made of observed ATPase in the recombined system with that expected from the measured activities in the separated fractions. The recombined systems generally contained more S-ATPase and less I-ATPase activity than expected. However the magnitude of the effects was small and further work is clearly necessary before a definite conclusion can be reached.

DISCUSSION

One of the premises upon which these experiments were based is that only a small fraction of the membrane units are involved in active transport and ATPase activity. This premise was based on two lines of reasoning. First, Glynn (15) has shown that only 600 molecules of ouabain per human red cell are sufficient to reduce the rate of active transport by one-half. Similar concentrations of cardiac glycoside are sufficient to inhibit active K-Na transport in sheep red cells. Thus, if it is assumed that one molecule of inhibitor can block only one transport site, there can be no more than 1000 to 3000 sites per cell. A second line of argument involves the actual rates of K-Na transport and Na + K–sensitive ATPase-catalyzed hydrolysis of ATP in sheep red cell membranes. The maximum rates for both these processes are about 0.6×10^{-3} M/liter RBC $\times$ hr. (7) or about 5000 K and Na ions transported and ATP molecules hydrolyzed per cell per second. Even if the transit time for a K or Na ion across the 100 A thickness of the membrane or the reaction time for ATP hydrolysis was as extraordinarily slow as 1 second, there would still be only 5000 sites per cell. The extreme slowness of this transit time can be seen from the fact that the average time required for a sodium ion in aqueous solution to diffuse 100 A is about 4×10^{-8} seconds while molecular relaxation times are in the same order of magnitude. If the transit time is faster than 1 second, even fewer than 5000 transport sites per cell would be required. The fact that most of the ATPase in the 20 minute HK sonicate was recovered in a density fraction which contained only 8 per cent of the membrane nitrogen is consistent with the idea that, like the active transport system, the enzyme(s) is located in relatively few membrane units. It is highly likely that further refinement of the density gradient will allow recovery of the enzymatic activity in a still smaller fraction of the membrane units.

The results obtained by separation of the components in the 3 minute sonicates according to density clearly establish that the Na + K–sensitive and much of the Na + K–insensitive ATPase activities are located in different membrane fragments. All the S-ATPase was recovered in large, light fragments whereas much of the I-ATPase was found in small, heavy fragments. Separation of these membrane components is associated with loss of Na + K–sensitive and gain of insensitive ATPase. The usual equality of lost sensitive and gained insensitive activity (Tables X and XI) suggests that the change involves conversion of one catalytic system from dependence on Na + K to independence of these ions. It is not, of course, possible to rule out that some of the insensitive ATPase is a different enzyme system which can never acquire Na + K sensitivity and is unrelated to active transport. For example, sonication and separation of fragments in the centrifuge could destroy or inhibit S-ATPase while simultaneously activating an independent I-ATPase.

If the view that S-ATPase consists of a complex between membrane units containing the enzyme(s) itself and light, phospholipid-containing units is correct, it is reasonable to speculate that active K-Na transport may also involve the relation between such units. It should be emphasized that the extremely long time available for the transport process (*ca.* 1 second, see above) leaves completely unspecified the size of the membrane units which move during active transport. They could be very large and have a relatively long transit time or relaxation time (*e.g.*, $10^{-3}–10^{-2}$ second) or be small and have a more rapid transit or relaxation time (*e.g.*, $10^{-7}–10^{-6}$ second). Thus, movement of entire 200 A diameter plaques or even groups of plaques is by no means ruled out by present information. It is entirely possible that the "carrier" is such a large aggregate of macromolecules.

These data also have implications for the molecular basis of the genetic difference in K-Na transport in HK and LK sheep red cells. In particular they suggest that the difference may not involve the ATPase system itself but rather the molecules (possibly phospholipids) in adjacent membrane units which control formation of the Na + K–sensitive complex. If so, the difference in phospholipid composition must be quite subtle (*e.g.*, in specific fatty acids) since the amount and type of these molecules present in the two types of cells are identical (C. F. Reed, personal communication).

REFERENCES

1. Skou, J. C., The influence of some cations on an adenosine triphosphatase from peripheral nerves, *Biochim. et Biophysica Acta,* 1957, **23**, 394.

2. Dunham, E. T., and Glynn, I. M., Adenosine triphosphatase activity and the active movements of alkali metal ions, *J. Physiol.*, 1961, **156**, 274.

3. Post, R. L., Merritt, C. R., Kinsolving, C. R., and Albright, C. D., Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte, *J. Biol. Chem.*, 1960, **235**, 1796.

4. Hoffman, J. F., Cation transport and the structure of the red cell plasma membrane, *Circulation*, 1962, **26**, 1201.

5. Whittam, R., The asymmetrical stimulation of a membrane adenosine triphosphatase in relation to active cation transport, *Biochem. J.*, 1962, **84**, 110.

6. Tosteson, D. C., and Hoffman, J. F., Regulation of cell volume by active cation transport in high and low potassium sheep red cells, *J. Gen. Physiol.*, 1960, **44**, 169.

7. Tosteson, D. C., Active transport, genetics and cellular evolution, *Fed. Proc.*, 1963, **22**, 19.

8. Evans, J. V., King, J. W. B., Cohen, B., Harris, H., and Warren, F. L., Genetics of hemoglobin and blood potassium differences in sheep, *Nature*, 1956, **178**, 849.

9. Hillier, J., and Hoffman, J. F., On the ultrastructure of the plasma membrane as determined by electron microscopy, *J. Cell. and Comp. Physiol.*, 1953, **42**, 203.

10. Rosett, T., Smashing cells by ultrasound, *New Scientist*, 1963, **22**, 366.

11. Bartlett, G. R., Phosphorus assay in column chromatography, *J. Biol. Chem.*, 1959, **234**, 466.

12. Marinetti, G. V., Chromotographic separation, identification and analysis of phosphatides, *J. Lipid Research*, 1962, **3**, 1.

13. Welt, L. G., McManus, T. J., Tosteson, D. C., and Wright, T. O., Membrane-bound ATP in erythrocyte ghosts, 18th Annual Meeting, Society of General Physiologists, Woods Hole, Sept. 4–7, 1963.

14. Tosteson, D. C., Aggregation of sheep red cell membrane fragments, data to be published.

15. Glynn, I. M., The action of cardiac glycoside on sodium and potassium movements in human red cells, *J. Physiol.*, 1957, **136**, 148.