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ASYMMETRIC INTERACTION OF INSIDE-OUT AND RIGHT-SIDE-OUT ERYTHROCYTE MEMBRANE VESICLES WITH OUABAIN

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SUMMARY

Inside-out and right-side-out human erythrocyte membrane vesicles were prepared as described (Steck, T. L., Weinstein, R. S., Strauss, J. H. and Wallach, D. F. H. (1970) *Science* 168, 255–257) and used to examine asymmetric properties of alkali cation-sensitive ATPase activity.

1. The Na^+- or (Na^+–K^+)-ATPase (ATP phosphohydrolase, EC 3.6.3.1) of inside-out vesicles was relatively insensitive to ouabain inhibition whereas the Na^+- or (Na^+–K^+)-ATPase of right-side-out vesicles and ghosts was markedly ouabain sensitive.

2. Ouabain added during vesiculation resulted in inside-out vesicles devoid of Na^+- or (Na^+–K^+)-ATPase activity, whereas right-side-out vesicles retained $\geqslant$ 70% of the activity observed without ouabain treatment.

3. Specific [^{3}H]ouabain binding by inside-out vesicles was negligible, whereas erythrocyte ghosts and right-side-out vesicles bound approximately 1.1 and 0.7 pmoles/mg protein, respectively. A direct relation between the degree of ouabain binding to the membranes and the degree of ouabain inhibition of (Na^+–K^+)-ATPase was observed.

4. These results provide further direct evidence that (i) ouabain binding sites are located on the outer membrane surface and (ii) 'inside-out' vesicles are indeed inside-out with respect to the sidedness of the ouabain-binding sites.

INTRODUCTION

Steck *et al.*[1] have developed a technique for obtaining erythrocyte vesicles which appear to be of two types, inside-out and right-side-out. The procedure involves treatment of human erythrocyte ghosts with alkaline phosphate buffer of low ionic strength followed by shearing and centrifugation to equilibrium on dextran gradients. Inside-out vesicles are characterized by and differentiated from both right-side-out vesicles and ghosts by several criteria including (1) internalization of their sialic acid residues, (2) a distinct morphological appearance when examined by freeze–cleave electron microscopy and (3) rate of 1-anilinonaphthalene-8-sulfonate binding[1,2]. Furthermore, proteolytic digestion profiles of the major proteins of inside-out vesicles are different from the profiles of precursor ghosts or right-side-out vesicles[3].

Steck *et al.*[3] concluded that the erythrocyte membrane is composed of an asymmetric organization of proteins, some of which appear to penetrate from the outer to the inner surface.

In recent studies, we have taken advantage of the simple elegant procedure developed by Steck *et al.*[1] to examine the asymmetric membrane-bound ouabain-sensitive alkali cation-stimulated ATPase enzyme system. Although the asymmetry of (Na^+-K^+)-ATPase has been described in studies with intact and resealed erythrocytes, these latter preparations lack direct accessibility to nucleotide substrates; in this respect, inside-out preparations should be particularly advantageous, *i.e.* they should retain both the asymmetry of the original cell membrane and allow direct accessibility of substances to the 'inside' surface. The present report shows (1) specific ouabain binding and inhibitory sites are restricted to the interior in inside-out vesicles and that these vesicles are indeed inside-out and (2) serves to directly confirm previous studies with giant squid axons and human red cell ghosts which indicated that ouabain acts only on the external surface of the membrane[4,5].

METHODS

Inside-out and right-side-out membrane vesicles were prepared by the method of Steck *et al.*[1] with minor modifications. The hemoglobin-free red cell ghosts underwent spontaneous vesiculation in alkaline (pH 8.0) 0.5 mM Na_2HPO_4 buffer. The vesicles were sheared from the ghosts by 3–5 passes through a 27-gauge needle (1.5 inch) and were separated by isopycnic dextran gradient centrifugation for 16 h at $100000 \times g$. Vesicles in the two major zones, inside-out vesicles at 1.01–1.03 g/cm^3 and right-side-out vesicles at 1.05–1.065 g/cm^3 were removed separately from the gradients by syringe and were freed of dextran by washing with 20 mM Tris–glycylglycine in 0.1 mM $MgCl_2$ or $MgSO_4$ (pH 7.4) (Solution G) as follows; the vesicle suspensions in dextran were diluted (10-fold) by gradual addition of 10 mM Tris–glycylglycine in 0.1 mM $MgCl_2$ or $MgSO_4$ (pH 7.4), allowed to equilibrate 10 min at 2 °C, and collected by centrifugation at 13000 rev./min for 20 min at 2 °C. The sedimented vesicles were washed 1 or 2 times with 20 mM Tris–glycylglycine in 0.1 mM $MgCl_2$ or $MgSO_4$ (pH 7.4) and resuspended in the same buffer to a final protein concentration of 0.5–1.0 mg/ml. The homogenate (unseparated vesicles after shearing) was allowed to stand overnight at 4 °C in 0.5 mM Na_2HPO_4 (pH 8.0) containing 0.1 mM $MgCl_2$ or $MgSO_4$ (1.5 mg homogenate protein/ml) and was then washed and resuspended in the same manner as the vesicles. Washed red cell ghosts were allowed to stand overnight at 4 °C in 5 mM Na_2HPO_4 (pH 8.0) (original wash solution, 1.5 mg ghost protein/ml) and were washed and resuspended in the same manner as the vesicles.

ATPase activities were measured using [γ-^{32}P]ATP as previously described[6,7]. The assay medium contained 0.1 ml membrane suspension, 40 μM $MgCl_2$ or $MgSO_4$ and 8 mM Tris–glycylglycine. The final pH was 7.4 and the temperature 37 °C. Assays were carried out for 5 min at 2 μM ATP or 10 min at 40 μM ATP. The rate of ATP hydrolysis was determined with either (a) 40 mM KCl or (b) 40 mM NaCl or (c) 35 mM NaCl *plus* 5 mM KCl. ATP hydrolysis in the presence of 40 mM KCl is referred to as Mg^{2+}-ATPase activity. With 2 μM ATP the difference

b−a is referred to as Na^+-ATPase activity and with 40 μM ATP the difference c−a is referred to as (Na^+–K^+)-ATPase activity.

The binding of [^{3}H]ouabain was determined as described by Hoffman[8] with slight modification. To determine total ouabain binding, ghosts or vesicles were incubated for 30 min at 37 °C in a final volume of 1.0 ml in a medium which contained 0.1 mg ghost or vesicle protein, 2 mM ATP, 40 mM NaCl, 1.25 mM $MgCl_2$, 0.25 mM EDTA, 20 mM Tris–glycylglycine (pH 7.4) and [^{3}H]ouabain at various concentrations as indicated in the text. Nonspecific binding of [^{3}H]ouabain was estimated by either adding excess ouabain (0.1 mM) or by omitting ATP from the reaction medium. Specific ouabain binding refers to total binding *minus* nonspecific binding. The reaction was terminated by placing the tubes in an ice-bath and the tubes were centrifuged at 13000 rev./min for 20 min at 2 °C. The supernatants were discarded and the pellets were washed 2 or 3 times with 1.5 ml of a solution containing 40 mM NaCl, 1.25 mM $MgCl_2$, 0.25 mM EDTA and 20 mM Tris–glycylglycine (pH 7.4). The resulting pellets were solubilized with 0.5 ml 0.1 M NaOH. Aliquots of 0.3 ml and 0.1 ml were taken for determinations of radioactivity and protein, respectively.

Sialic acid was determined by the method of Warren[9]. Total sialic acid was the amount released following incubation of the washed membrane fractions with 0.05 M H_2SO_4 at 80 °C for 1 h. Neuraminidase-sensitive sialic acid was the amount released after incubation of ghosts or vesicles with neuraminidase (40 μg/mg membrane protein) in 0.1 M Tris–acetate buffer (pH 5.7) for 1 h at 37 °C[10]. *N*-Acetylneuraminic acid was used as a standard for the color assay.

Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter using a modified Bray's solution. In the [^{3}H]ouabain binding experiments quenching was approximately 10% as determined by both external and internal standard methods. Quenching was routinely determined and corrected for by the external standard method.

Water, ATP and dextran spaces were determined simultaneously in a medium similar to the one used for ATPase determinations and which contained in 1.0 ml, 0.2–0.3 mg vesicle protein; 8 μmoles Tris–glycylglycine (pH 7.4); 40 μmoles KCl; ^3H_2O, $2.1 \cdot 10^6$ cpm; [*carboxyl*-^{14}C]dextran (mol. wt 60000–90000), 0.2 mg containing $3.05 \cdot 10^5$ cpm; dextran (average mol. wt 72000), 1.0 mg; 50 or 200 nmoles [γ-^{32}P]ATP containing $6.0 \cdot 10^5$ cpm; $MgSO_4$ at a concentration equal to the ATP concentration. A cpm ratio of 7:1:2 for ^3H_2O: [*carboxyl*-^{14}C]dextran:[γ-^{32}P]ATP was used. In experiments where the citrate space was determined, [1,5-$^{14}C_2$]citrate (1 μmole containing $3.0 \cdot 10^5$ cpm) replaced the dextran. The vesicles were incubated for 2 min at 37 °C, placed in ice and centrifuged at 13000 rev./min for 30 min at 2 °C. The supernatants were removed and a 0.2 ml aliquot diluted to 3.0 ml with 5% trichloroacetic acid and 0.1 ml counted in 10 ml Aquasol. The tubes containing the vesicle pellets were inverted, allowed to drain, and excess moisture removed from the walls of the tubes by blotting. The pellets were extracted at room temperature with 0.5 ml 5% trichloroacetic acid solution containing 2.5 mM ATP and 5 mM phosphate for 15 min with intermittent shaking. The pellet suspensions were centrifuged for 30 min at 13000 rev./min and 0.1 ml of the resulting supernatant counted in 10 ml Aquasol. Channel settings of the Packard Tri-Carb spectrometer were determined so that the ^{3}H radioactivity was confined to one channel (Red, 100%

amplification, window settings 50–1000), 34% of the ^{14}C radioactivity (Green channel, 10% amplification, window settings 220–1000) appeared in the ^{3}H channel and 16% and 3% of the ^{32}P radioactivity (Blue channel, amplification 1.4%, window settings 130–1000) appeared in the ^{3}H and ^{14}C channels, respectively. Both internal and external standard counting indicated negligible quenching. The water in the vesicle pellet (water space) was calculated from the following equation:

$$\text{Water space } (\mu l) = \frac{\text{total cpm } {}^{3}H_2O \text{ in pellet}}{\text{cpm } {}^{3}H_2O \text{ in supernatant per } \mu l \text{ of supernatant}} \quad (1)$$

and the ATP, dextran and citrate impermeable spaces were calculated as shown below for the ATP impermeable space:

$$\text{ATP permeable space } (\mu l) = \frac{\text{total cpm } [\gamma\text{-}{}^{32}P]ATP \text{ in pellet}}{\text{cpm } [\gamma\text{-}{}^{32}P]ATP \text{ in supernatant per } \mu l \text{ of supernatant}} \quad (2)$$

$$\text{ATP impermeable space } (\mu l) = \text{water space } (\mu l) - \text{ATP permeable space } (\mu l) \quad (3)$$

The results were then expressed on the basis of the protein content of the vesicles.

Protein was determined by the method of Lowry *et al.*[11] with crystalline bovine serum albumin as a standard. $[\gamma\text{-}{}^{32}P]ATP$ was prepared as previously described[6] Ouabain, *N*-acetylneuraminic acid, neuraminidase from *Clostridium perfringens*, dextran (average mol. wt 72000) and crystalline bovine serum albumin were purchased from Sigma. [*carboxyl*-^{14}C] Dextran, mol. wt 60000–90000 (1.28 mCi/g) and $^{3}H_2O$ (1 mCi/ml) and Aquasol were purchased from New England Nuclear and [1,5-$^{14}C_2$]citrate (6 mCi/mM) from the Radiochemical Centre (Amersham). [^{3}H]ouabain was obtained from New England Nuclear (12 Ci/mM) and the concentrations checked spectrophotometrically at 220 nm (molar extinction coefficient $1.54 \cdot 10^{4}$ moles) as described by Lauf *et al.*[12].

RESULTS

Preliminary experiments were carried out to check the membrane asymmetry of the vesicles by examining the accessibility of their sialic acid residues to enzymatic hydrolysis by neuraminidase since the sialic acid of red cells is located on the outer surface of the membrane[13]. Total sialic acid and the percentage released by neuraminidase are shown in Table I. In agreement with the results obtained by Steck *et al.*[1], only 22% of the sialic acid residues of inside-out vesicles were accessible to enzymatic attack by neuraminidase. In contrast, neuraminidase released 86% of the sialic acid residues of 'normally' orientated ghosts and 97% of the sialic acid residues of right-side-out vesicles. The moderate hydrolysis (59%) by neuraminidase of the sialic acid residues of the homogenate, which is a mixture of unseparated inside-out and right-side-out vesicles, reflected the heterogeneity of that fraction.

Ouabain sensitivity

The sensitivity of membrane-bound ATPase to ouabain inhibition was a parameter used to characterize the membrane orientation of inside-out and right-side-out vesicles with respect to ouabain binding sites. Previous studies at low ATP concentrations (2 μM) have shown that Na^{+} alone stimulates ATP hydrolysis of

TABLE I

NEURAMINIDASE-SENSITIVE SIALIC ACID OF GHOSTS AND VESICLES

Results are expressed as the mean ± S.D. for three separate vesicle preparations. The numbers in parentheses indicate the number of determinations.

Fraction	*Total sialic acid (nmoles/mg protein)*	*Percentage released by neuraminidase*
Ghosts	145 ± 42(4)	86 ± 15(4)
Homogenate	169 ± 47(4)	51 ± 15(4)
Inside-out vesicles	176 ± 61(4)	22 ± 8(4)
Right-side-out vesicles	151 ± 19(4)	97 ± 25(3)

TABLE II

OUABAIN SENSITIVITY OF Na^+-ATPase

Na^+-ATPase activity is expressed as pmoles $^{32}P_i$ released/mg protein per min. Results are expressed as the mean ± S.D. or as the range. Numbers in parentheses indicate the number of individual experiments with separate vesicle preparations. Na^+-ATPase activity was measured using 2 μM [γ-^{32}P]ATP as described in Methods.

Fraction	*Na^+-ATPase activity*	*Inhibition (%)*	
		20 μM ouabain	*200 μM ouabain*
Ghosts	231 ± 43(4)	51 , 74(2)	65–100(3)
Inside-out vesicles	193 ± 101(6)	17 ± 10(6)	15–47(3)
Right-side-out vesicles	224 ± 110(6)	89 ± 10(5)	88,91(2)

human erythrocyte ghosts[14]; the increment in the presence of Na^+ has been termed Na^+-ATPase. The data in Table II indicate that the Na^+-ATPase activity of ghosts and of right-side-out vesicles was markedly inhibited by 20 μM ouabain. In contrast, Na^+-ATPase activity of inside-out vesicles was relatively insensitive to inhibition by both 20 μM and 200 μM ouabain whereas right-side-out vesicles were almost completely inhibited. The Na^+-ATPase activity of ghosts and both types of vesicles varied somewhat from preparation to preparation as shown in Table II. At 40 μM ATP both Na^+ and K^+ are required for maximal ATPase activity[14]. As shown in Table III, the pattern of ouabain-sensitive (Na^+–K^+)-ATPase activity of ghosts, inside-out and right-side-out vesicles was similar to that observed for Na^+-ATPase activity. With 20 μM ouabain, inhibition of inside-out vesicles was 12%, that of right-side-out vesicles, 73%; with 200 μM ouabain, inhibition of inside-out vesicles was 31%, that of right-side-out vesicles, 83%. In a time-course experiment at 40 μM ATP, the (Na^+–K^+)-ATPase activity of inside-out vesicles was linear for at least 20 min and was inhibited 13%, 17% and 8% by 20 μM ouabain at 5, 10 and 20 min, respectively. Variation in the level of (Na^+–K^+)-ATPase activity from preparation to preparation was also observed as indicated in Table III. This considerable variation is similar to that noted by Schrier *et al.*[15] with vesicles obtained following

TABLE III

OUABAIN SENSITIVITY OF (Na^+–K^+)-ATPase

(Na^+–K^+)-ATPase activity is expressed as pmoles $^{32}P_i$ released/mg protein per min. Results are expressed as the mean ± S.D. Numbers in parentheses indicate the number of individual experiments with separate vesicle preparations. (Na^+–K^+)-ATPase activity was measured using 40 μM [γ-^{32}P]ATP as described in Methods.

Fraction	*(Na^+–K^+)-ATPase activity*	*Inhibition (%)*	
		20 μM ouabain	*200 μM ouabain*
Ghosts	793 ± 278(7)	99 ± 28(6)	83 , 140(2)
Inside-out vesicles	450 ± 278(9)	12 ± 9(9)	31 ± 15(7)
Right-side-out vesicles	468 ± 182(9)	73 ± 11(8)	83 ± 5(4)

TABLE IV

EFFECTS OF OUABAIN ADDITION AT START OF VESICULATION PERIOD

A final concentration of 65 μM ouabain was present as indicated when red cell ghosts were undergoing vesiculation. Inside-out and right-side-out vesicles were separated, washed and resuspended in the routine manner as described in Methods. 20 μM ouabain was present in the ATPase assay medium as indicated. Activity is expressed as pmoles $^{32}P_i$ released/mg protein/min.

Assay conditions		*Inside-out vesicles*		*Right-side-out vesicles*	
		Activity	*Inhibition(%) with 20 μM ouabain in assay medium*	*Activity*	*Inhibition (%) with 20 μM ouabain in assay medium*
Na^+-ATPase at 2 μM ATP	Control	103	0	113	88
	65 μM ouabain in vesiculation medium	8	0	81	91
(Na^+–K^+)-ATPase at 40 μM ATP	Control	213	0	216	89
	65 μM ouabain in vesiculation medium	0	–	170	118

shearing in a French press; this may be related to variable loss of protein during vesicle formation[16].

To ascertain whether the ouabain binding sites of inside-out vesicles are functional but inaccessible, attempts were made to 'trap' ouabain during vesiculation. In the representative experiment shown in Table IV, 65 μM ouabain was added at the beginning of the 1-h vesiculation period. Following centrifugation, shearing and density gradient centrifugation, inside-out and right-side-out vesicles were separated

and washed as described in Methods, and their ATPase activities measured. Ouabain in the vesiculation medium resulted in inside-out vesicles devoid of either Na^+- or (Na^+-K^+)-ATPase activity. In contrast, right-side-out vesicles formed with ouabain added to the vesiculation medium retained 72% of the Na^+-ATPase and 79% of the (Na^+-K^+)-ATPase activity of untreated right-side-out vesicles. Furthermore, the Na^+- and (Na^+-K^+)-ATPase activities of right-side-out vesicles formed in the presence of ouabain are completely inhibited by ouabain subsequently added to the assay medium.

To test further whether ouabain was enclosed in inside-out vesicles, an experiment (not shown) was carried out with [^{3}H]ouabain added at the start of the vesiculation period. The suspensions were centrifuged, sheared and washed until the supernatants were free of radioactivity (twice with 0.5 mM Na_2HPO_4 in 0.1 mM $MgSO_4$, pH 8.0), layered on dextran gradients and the vesicles isolated. With 60 μM [^{3}H]ouabain (63880 cpm/ml) inside-out vesicles retained 2218 pmoles/mg, right-side-out vesicles only 220 pmoles/mg. When 60 μM [^{3}H]ouabain was added to the sheared vesicles (homogenate) and the mixture then layered on gradients, only 323 pmoles [^{3}H]ouabain/mg protein was retained in the inside-out vesicles. This experiment provided further evidence that ouabain was retained by inside-out vesicles.

[^{3}H]Ouabain binding

Since the ATPase activity of inside-out vesicles is relatively insensitive to inhibition by ouabain, [^{3}H]ouabain binding was measured to determine whether the glycoside was bound to the vesicles but could not inhibit their alkali cation-sensitive ATPase activity. Specific ouabain binding was measured as described in Methods. As shown in Table V, specific [^{3}H]ouabain binding by inside-out vesicles was negligible at the two ouabain concentrations used. In contrast, with $4.4 \cdot 10^{-8}$ M ouabain, ghosts bound 1.07 pmoles/mg protein and right-side-out vesicles bound somewhat less (0.68 pmole/mg protein). Increasing the ouabain concentration to $1.9 \cdot 10^{-7}$ M did not increase the specific [^{3}H]ouabain binding. The [^{3}H]ouabain bound (1 pmole/mg) by the ghost preparation is similar to the amount of phosphorylated intermediate reported previously for erythrocyte membrane fragments[14].

TABLE V

[^{3}H]OUABAIN BINDING BY GHOSTS, INSIDE-OUT AND RIGHT-SIDE-OUT VESICLES

[^{3}H]ouabain binding was determined as described in Methods. The results shown are typical of four separate vesicle preparations.

Fraction	*Ouabain (M)*	*[^{3}H]Ouabain bound (pmoles/mg protein)*
Ghosts	$4.4 \cdot 10^{-8}$	1.07
	$1.9 \cdot 10^{-7}$	1.17
Inside-out vesicles	$4.4 \cdot 10^{-8}$	0.09
	$1.9 \cdot 10^{-7}$	0.09
Right-side-out vesicles	$4.4 \cdot 10^{-8}$	0.75
	$1.9 \cdot 10^{-7}$	0.68

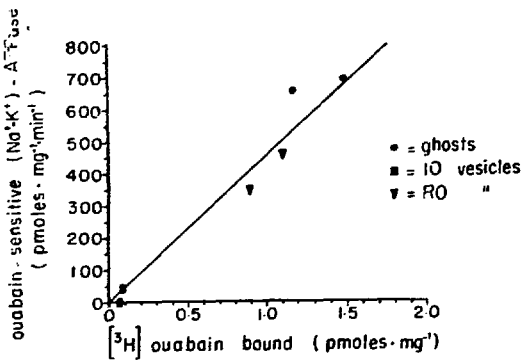

Fig. 1. The results shown are from two experiments carried out with separate vesicle preparations. $(Na^+–K^+)$-ATPase activity was measured using 40 μM [γ-^{32}P]ATP and [^{3}H]ouabain binding was determined as described in Methods. IO and RO vesicles, inside-out and right-side-out vesicles, respectively.

It was of interest to determine if the degree of ouabain binding correlated with the ouabain-sensitive component of the $(Na^+–K^+)$-ATPase activity. The results shown in Fig. 1 were obtained with two separate vesicle preparations. Inside-out vesicles bound little [^{3}H]ouabain and had low ouabain-sensitive $(Na^+–K^+)$-ATPase activity. The increased amounts of [^{3}H]ouabain bound to right-side-out vesicles and red cell ghosts were directly proportional to the increased ouabain-sensitive $(Na^+–K^+)$-ATPase in these fractions. Thus, there is a direct relationship between the amount of [^{3}H]ouabain bound and the ouabain-sensitive $(Na^+–K^+)$-ATPase activity.

ATP permeability

Experiments aimed to measure ATP permeability were carried out by incubating vesicles for 2 min at 37 °C in varying concentrations of equimolar $MgSO_4$ and ATP in the same medium used to determine Mg^{2+}-ATPase activity (40 mM KCl added) using ^3H_2O, [γ-^{32}P]ATP and [*carboxyl*-^{14}C]dextran or [1,5-$^{14}C_2$]citrate

TABLE VI

PERMEABILITY OF INSIDE-OUT AND RIGHT-SIDE-OUT VESICLES TO ATP, DEXTRAN AND CITRATE

Results are expressed as the mean ± S.D. for three separate vesicle preparations. Numbers in parentheses indicate the number of individual determinations. Water spaces, ATP-impermeable and dextran- or citrate-impermeable spaces were determined simultaneously using ^3H_2O, [γ-^{32}P]-ATP and [*carboxyl*-^{14}C]dextran or [1,5-$^{14}C_2$]citrate as described in Methods.

Fraction	*Water space (µl/mg protein)*	*% water space*		
		Dextran-impermeable space	*Citrate-impermeable space*	*ATP-impermeable space*
Inside-out vesicles				
50 µM ATP	36.9 ± 3.5(8)	28.6 ± 11.3(4)	21.6 ± 5.2(4)	25.2 ± 10.2(8)
200 µM ATP	35.9 ± 2.4(8)	30.1 ± 7.0(4)	21.8 ± 4.4(4)	28.2 ± 8.1(8)
Right-side-out vesicles				
50 µM ATP	88.3 ± 16.1(8)	15.9 ± 11.1(4)	14.9 ± 7.1(4)	15.4 ± 8.7(8)
200 µM ATP	90.4 ± 16.1(8)	13.9 ± 8.4(4)	8.4 ± 11.3(4)	12.7 ± 8.5(8)

as described in Methods. The results in Table VI indicate that under the conditions used approximately 25% of the water space of the inside-out vesicle pellet was impermeable to ATP at either 50 μM or 200 μM ATP. The inside-out vesicle pellet also had a dextran-impermeable space (30% of the water space) and a citrate-impermeable space (22% of the water space). The dextran spaces were not affected by varying the dextran concentration (not shown). In comparison, the water space of the right-side-out vesicle pellet was approximately 2.5 times greater than the water space of the inside-out vesicle pellet. The variability in the ATP, dextran and citrate impermeable spaces of the right-side-out vesicles preclude any conclusion regarding their permeability to these substances.

DISCUSSION

The asymmetry of the (Na^+–K^+)-ATPase system has been investigated previously using erythrocyte ghosts resealed with various media. These previous studies showed that K^+ activates at the outer surface of the cell membrane, whereas Na^+ activates at the inner surface of the cell membrane[17,18] and inorganic orthophosphate is released inside the cell[19,20]. Detailed studies of the (Na^+–K^+)-activated ATPase system, in particular the associated partial reactions such as the [^{14}C]ADP–ATP exchange and membrane phosphorylation by [γ-^{32}P]ATP, have been limited to preparations with direct access to the nucleotide substrates. In the past, only fragmented membranes fulfilled this prerequisite. The development of a relatively simple technique for preparing normally oriented and inverted membrane vesicles, should provide a particularly useful means of further probing the nature of this multistage reaction system, provided that the original asymmetry is indeed that of the intact cell and that sufficient impermeability can be attained.

In these experiments the ghosts and right-side-out vesicles were relatively permeable to ATP under the conditions used as shown by their ability to catalyze ATP hydrolysis without further disruption. Although permeability of inside-out vesicles to ATP cannot be evaluated from ATPase activity measurements, their limited permeability is suggested by their relative insensitivity to ouabain inhibition and binding and by their relatively large ATP-impermeable space.

That the ouabain-binding sites are functional but inaccessible on inside-out vesicles has been demonstrated by the experiments showing that addition of ouabain during vesiculation inhibited completely the Na^+- or (Na^+–K^+)-ATPase activity of the vesicle fraction appearing in the dextran gradient in the location of inside-out vesicles. It is unlikely that ouabain was actually bound to the vesicles under the conditions of the vesiculation process, *i.e.* 0 °C (ref. 14), and absence of Mg^{2+}, but probable that ouabain is 'trapped' inside the inside-out vesicles, and binds only under the appropriate conditions, *e.g.* during the ATPase assay, with ATP, Mg^{2+} and Na^+ present and the temperature at 37 °C. In previous studies with erythrocyte membrane fragments, ouabain inhibition of Na^+-ATPase activity measured at 0 °C could be observed only after preincubation of the membranes with ouabain at 37 °C (ref. 14). Moreover, since most (75%) of the Na^+- or (Na^+–K^+)-ATPase activity of right-side-out vesicles is observed following their isolation from the sheared, ouabain-treated ghosts, conditions prior to the ATPase assay are probably unfavourable for ouabain binding.

From the data in Tables II, III, and V and the assumption that either one phosphorylation site or one ouabain binding site is equivalent to one catalytic unit, the catalytic centre activity for erythrocyte ghosts and right-side-out vesicles can be calculated. The values obtained at 2 μM ATP (Na^+-ATPase), approximately 250 per min, are similar to those reported previously for erythrocyte membrane fragments[14]; however, values at 40 μM ATP (Na^+–K^+)-ATPase, 700 per min, were about one-third that estimated previously[14]. This discrepancy may be due to either a kinetic alteration associated with the preparation of ghosts and right-side-out vesicles or limited permeability to K^+ and requires further investigation.

The relative insensitivity of inside-out vesicles to ouabain inhibition and binding provide direct evidence for (1) the asymmetry of the ouabain binding site, (2) the retention of this asymmetry upon formation of inverted membrane vesicles and substantiate the direct relationship between the measurement of specific ouabain binding to the membranes and inhibition of the (Na^+–K^+)-ATPase.

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Since submission of our manuscript, Kant and Steck[21] provided further evidence that the top band fraction derived from ghosts in the absence of Mg^{2+} (see Methods and ref. 1) are impermeable inside-out vesicles. However, they have raised doubts concerning the impermeability and nature of the fraction which bands at the higher density and referred to as right-side-out vesicles. In our study, the questionable presence of a solute-impermeable space and the accessibility of ATP to (Na^+–K^+)-ATPase of this latter fraction are in accord with their observations.

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