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AN IMPROVED PROCEDURE FOR THE PREPARATION AND MEASUREMENT OF $(Na^+ + K^+)$-ATPase IN HUMAN ERYTHROCYTES

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Sodium and potassium-stimulated adenosine triphosphatase ($(Na^+ + K^+)$-ATPase) activity and membrane permeability as measured by ^{22}Na influx were compared in erythrocyte membranes prepared by hypotonic and detergent (ionic and non-ionic) haemolysis. The detergent-treated erythrocytes showed significantly ($P < 0.001$) greater $(Na^+ + K^+)$-ATPase and permeability to ^{22}Na compared with erythrocyte membranes prepared by hypotonic haemolysis. In addition, increased enzyme activities were exhibited when membranes initially prepared by hypotonic haemolysis were subsequently exposed to 1% (w/v) saponin in the reaction mixture. A maximum level of $(Na^+ + K^+)$-ATPase was achieved with 1.5 mg/ml sodium deoxycholate haemolysing agent. A method for erythrocyte vesicle preparation with sodium deoxycholate as haemolysing agent was developed. This method yielded high and reproducible $(Na^+ + K^+)$-ATPase activity, which could be related to a new additional index of vesicle count. Biological variations and dietary habits influenced day to day levels of $(Na^+ + K^+)$-ATPase activity but these fluctuations were kept to a minimum when blood samples were collected under identical conditions after an overnight fast, and a strictly standardized protocol of erythrocyte vesicle preparation was followed. Reproducibility of $(Na^+ + K^+)$-ATPase activity from assay to assay could be predicted by monitoring the completeness of haemolysis, vesicle size-distribution graphs, vesicle count and vesicular protein concentrations. The initial packed erythrocyte volume and the enzyme assay volume yielded the most reliable indices of $(Na^+ + K^+)$-ATPase activity, though vesicle count could also be used as an index.

Introduction

For about 30 years erythrocytes have served as a useful model of the more inaccessible cells for cation transport and ATPase. However, a survey of the literature on extraction and measurement of ATPases shows that a large variety of protocols are used [1–6]. Conflicting data on erythrocyte $(Na^+ + K^+)$-ATPase have been reported in various disorders [7–10], and even when a similar method of hypotonic haemolysis was used for extracting the enzyme, normal values reported ranged from 0.05 to 0.29 μmol P_i/h per mg membrane-bound protein [11–14].

Our experience with the current methods showed large variations in $(Na^+ + K^+)$-ATPase between preparations of the same source. These variations were substantially reduced by a strictly standardized procedure for membrane preparation and improvements in protein and phosphate measurements. However, differences in $(Na^+ + K^+)$-ATPase levels still remained greater than 10% even when erythrocytes were obtained from the same source, and assayed on different days or prepared on separate occasions. The possible reasons for

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Abbreviation: EGTA, ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

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these differences are variable erythrocyte haemolysis between preparations; variable membrane permeability of prepared vesicles to substrate and activators, and variable extraction of ATPase components from erythrocytes. Day to day variations may also be biological in nature even though erythrocytes have a long life span (about 120 days) and possess no known capability to manufacture ATPase. Jørgensen and Skou [15] studying the effects of deoxycholate on ($Na^+ + K^+$)-ATPase suggested that increased yields were due to greater membrane permeability as a result of detergent action. Observations made by other workers [5, 16–19] also support this hypothesis. Thus the use of detergents could provide an avenue leading to high and reproducible levels of erythrocyte ($Na^+ + K^+$)-ATPase from one preparation to the next. The aim of this study was to establish a procedure of erythrocyte membrane preparation which would yield high and reproducible ($Na^+ + K^+$)-ATPase levels and would also provide reliable indices of ATPase activity.

Materials and Methods

Solutions

All reagents were AnalaR grade and double-distilled water was used throughout.

Solution I. Histidine-buffered isotonic saline containing in mmol/l: histidine 3.0; NaCl 155.0; adjusted to pH 7.4 (4°C) with dilute HCl.

Solution II. Erythrocyte membrane wash solution containing in mmol/l: EDTA 2.0; Imidazole 50.0; and 3% (w/v) sucrose; adjusted to pH 7.4 (20°C) with dilute HCl.

Solution III. Erythrocyte ATPase assay medium containing in mmol/l: NaCl 90.0; KCl 10.0; $MgCl_2$ 5.0: NaCN 10.0; Tris 30.0: EGTA 0.1; adjusted to pH 7.4 (4°C) with concentrated HCl. Where addition of ouabain was required Strophanthidin-G (Sigma Chemical Co.) was dissolved in the assay medium to give a final concentration of 0.1 mmol/l.

Solution IV. Erythrocyte ATPase substrate adenosine 5′-triphosphate from Koch-Light Laboratories Ltd., Colnbrook, Bucks, was made up in solution III to a concentration of 20 mmol/l, and adjusted to pH 7.4 (4°C) with dilute NaOH.

Blood samples

Fresh blood was obtained by venepuncture from normal healthy volunteers in the mornings. Blood (60 ml) was drawn into a heparinized (100 units/ml) syringe and transferred into chilled plastic bottles.

I. Preparation of erythrocyte membranes by hypotonic lysis. The following protocol was established after detailed comparisons between the various available methods were made (data not shown). Erythrocyte membranes were prepared essentially according to Dodge et al. [20] after the cells had been seperated from serum and leucocytes by three washes in solution I. Centrifugation at $1000 \times g$ for 5 min pelleted the erythrocytes; the serum and leucocytes were discarded after aspiration. The washed erythrocyte pellet was suspended in a volume of isotonic saline equal to the whole blood volume originally used. Haemoglobin was estimated (see below) and a blood cell count performed on an automated Coulter counter. Haemolysis was achieved by adding 10–30 vol. of hypotonic 10 imosM Tris-HCl (pH 7.5) solution containing 1 mM Tris-EDTA to 2 ml erythrocytes. After vigorous mixing for 5 min the haemolysate was centrifuged on a Super 25 (MSE) at $20000 \times g$ for 1 h at 4°C and the pellet was resuspended in 5 mM Tris-HCl (pH 7.5). This procedure was repeated until the supernatant was colourless; usually four times. The final membrane pellet was taken up in twice its volume of solution III without ouabain to contain an avarage of 3 mg protein/ml. The membrane suspension was either assayed on the same day or stored at 4°C until required.

II. Preparation of erythrocyte membranes by detergent haemolysis. The whole blood was washed three times by repeated centrifugation at 2000 rpm on a bench centrifuge for 5 min and resuspended in solution I. The washed red cell pellets were resuspended in solution II to yield a 10% cell suspension. To the red cell suspensions 1.5 mg/ml deoxycholate was added, unless otherwise stated, and incubated for 90 min with continual agitation. The resulting haemolysate was washed free of haemoglobin by three centrifugations at $20000 \times g$ for 40 min at 4°C on an MSE 25 centrifuge and resuspensions to 30 ml volume in 40 ml aluminium capped polycarbonate tubes. The resulting erythrocyte vesicle pellets were resuspended in

solution II by gentle inversion. The final washed vesicle pellet was taken up into one-third original blood volume solution III by mild dispersion with a 1 ml automated 'Finnpipette'. The vesicle suspension was examined on a Coulter counter and channelyzer.

Determination of membrane-bound protein

Protein concentration was assayed by Hartree's [21] modification of the method of Lowry et al. [22]. Membrane-bound protein was initially precipitated with 12% (w/v) trichloroacetic acid, centrifuged at 2000 rpm for 5 min and then the pellet resuspended in 0.9 ml solution A (7 mM potassium-sodium tartrate, 0.94 M Na_2CO_3, 0.5 M NaOH). The reaction was aided by incubation at 50°C for 10 min and then 0.1 ml solution B (70 mM potassium-sodium tartrate, 40 mM $CuSO_4 \cdot 5H_2O$, 0.1 M NaOH) was added and further incubated at ambient temperature for 10 min. 3 ml Folin-Ciocalteu's phenol reagent (1 ml diluted to 16 ml with distilled water) was rapidly added to ensure mixing and incubated at 50°C for 10 min. The absorbance at 655 nm was measured on a Pye Unicam SP 1800 spectrophotometer and protein calculated from a calibration curve of bovine serum albumin. Hartree's modification was used because a linear photometric response to protein (0–200 μg/ml bovine serum albumin) was obtained with an increase of 140% in sensitivity compared to the method of Lowry (data not shown).

Estimation of ^{22}Na influx

Two chilled 1 ml membrane samples in solution III, one with 0.1 mM ouabain were pre-equilibrated to 37°C for 15 min. At zero-time ^{22}Na (5 μCi/ml) was added to the first sample and at 30 s intervals 100 μl were removed. Each 100-μl aliquot was then filtered through a multihead filtering system fitted with 0.45 μm pore diameter cellulose acetate filters (Millipore Corporation, Bedford, MA 01730). Two 3-ml aliquots of chilled solution III were used to wash away any excess ^{22}Na not taken into the vesicles. Each filter was allowed to dry and then removed from the multihead and placed into stoppered tubes. The radioactivity was detected on an LKB gamma counter for either 10 min or 10000 counts and the mean of two counts was used. The above procedure was repeated on the other 1 ml membrane sample containing 0.1 mM ouabain.

Measurement of $(Na^+ + K^+)$-ATPase

180 μl of the membrane suspension (4°C) was added to either 270 μl solution III without ouabain or to 270 μl solution III with 0.1 mM ouabain. The enzyme activity was initiated by the addition of 50 μl 20 mM ATP (2 mM final) and the samples incubated at 37°C for 60 min. The reaction was stopped by the addition of 500 μl of a 'P_i-complexing chromophore' prepared essentially according to Lin and Morales [23]. After 10 min the extinction was measured at 420 nm on a spectrophotometer (Pye Unicam SP1800, Cambridge). Preliminary work had shown that a linear photometric response over a P_i concentration between 0 and 1 μmol/ml was achieved with Lin and Morales 'chromophore' and sensitivity increased by 2–3-fold compared to the method of Fiske and SubbaRow [24].

Estimation of haemoglobin

Using an automatic dilutor haemoglobin was converted to cyanmethaemoglobin (1:20) and the absorbance measured in a precalibrated LK 450 spectrophotometer. Haemoglobin was expressed as g/100 ml of original solution.

Sizing and counting of erythrocyte vesicles

Vesicle count and size-distribution graphs were made with a Coulter counter, Model ZFl and Coulter channelyzer (Coulter Electronics, Luton, U.K.), respectively. The Coulter counter detects particles by measuring changes in the conductivity of the electrolyte medium used to suspend the particles. The electrolyte medium was obtained from Coulter Electronics as azide-free balanced electrolyte solution, 'ISOTON II'. The particles are first passed through an orifice which allows only a single file of particles to pass through two electrodes. As each separated particle passes the electrodes a change in conductivity occurs and is recorded by the counter as a pulse. The amplitude of this pulse is directly proportional to the degree of resistance set up by the passing particle. The number of pulses are counted in a constant volume of 0.5 ml of the vesicle/particle suspension.

of resistance set up by the passing particle. The

cumulated by the Coulter channelyzer; each pulse is separated depending on its amplitude into one of its one hundred channels. A display of the distribution of these pulses is presented visually on a fluorescent screen and a permanent record of the displayed size-distribution curve can be obtained from a graph recorder attached to the channelyzer. From the size-distribution curve the mean particle size as well as an absolute particle count can be obtained. The count of particles in 0.5 ml vesicles suspension can be recorded on the Coulter counter. By determining the counts occurring underneath the main vesicle peak and expressing them as a fraction of the total count the absolute vesicle concentration can be determined.

Since we published our findings in obesity [25] we have further improved the vesicle counting by reducing significantly the background instrument 'noise'. By decreasing signal amplifications (Attenuation settings) and setting a constant current across the orifice electrodes sensitivity was maintained and counting of sub-vesicular particles (< 0.25 μm diameter) was reduced. The change in detection does not alter any relative differences presented in our previous findings since all analyses were performed on paired group samples. The Coulter channelyzer settings were modified from an attenuation ranging from 0.125 to 0.250 and set at 0.5 and the aperture setting set constant at 16. This effectively reduced the particles detected from a range of 10^4 to a range of 10^3 in samples diluted $4 \cdot 10^5$ fold. Thus, the $(Na^+ + K^+)$-ATPase levels remain the same when expressed as per ml erythrocytes, but are about 10-fold higher than before when related to the vesicle count.

Statistical analysis

Standard statistical methods were used. The coefficient of variance (C.V.) was used as a measure of variability from assay to assay and was derived from the following equation

$$\text{C.V.} = \frac{\text{standard deviation}}{\text{mean}} \cdot 100$$

Student's *t*-test was used to determine the significance.

Results

Interassay variability of $(Na^+ + K^+)$-ATPase from single sources of membranes prepared by hypotonic haemolysis

Initial studies on a single source of erythrocyte membranes prepared by hypotonic lysis with 10 imosM Tris-HCl had shown a large scatter of $(Na^+ + K^+)$-ATPase activities. When the $(Na^+ + K^+)$-ATPase was expressed per mg membrane-bound protein the coefficient of variance (C.V.) was 36% and 28.8% when related to assay volume (individual data not shown). By modifying the protocols for measuring P_i and protein concentra-

TABLE I

THE VARIABILITY OF $(Na^+ + K^+)$-ATPase ACTIVITY SUBSEQUENT TO STANDARDIZATION AND MODIFICATION OF THE PROCEDURES USED FOR MEASURING THIS ENZYME [3]

Samples were prepared using hypotonic haemolysis (see Methods) and were assayed on three occasions on the day after preparation. The indices, membrane-bound protein and assay volume (AV) for enzyme expression were compared. The ratio of erythrocyte volume to assay volume was kept constant so that assay volume is a reflection of the concentration of erythrocytes used. Each datum is the mean of triplicates with a within assay error of less than 5% coefficient of variance (C.V.). The mean of the C.V. values of each sample is an indication of the overall variability of the measurement and expression of $(Na^+ + K^+)$-ATPase.

Sample	μmol P_i/h per 0.5 ml AV	μmol P_i/h per mg membrane-bound protein
1	0.050	0.102
	0.047	0.196
	0.061	0.235
	C.V. (%) 13.9	C.V. (%) 38.4
2	0.113	0.314
	0.102	0.510
	0.097	0.285
	C.V. (%) 7.9	C.V. (%) 29.0
3	0.245	0.606
	0.240	0.622
	0.260	0.700
	C.V. (%) 4.2	C.V. (%) 7.8
Mean	C.V. (%) 8.7	25.1
Range (%)	4.2–13.9	7.8–38.4

tions and strict adherence to a standardized procedure for erythrocyte haemolysis (method 1) we were able to reduce the interassay variability of the $(Na^+ + K^+)$-ATPase from 36% to 25.1% (per mg membrane-bound protein) and from 20.8% to 8.7% when expressed as per ml assay volume (Table I).

Effect of saponin on erythrocyte membrane ($Na^+ + K^+$)-ATPase

Our initial studies on stored erythrocyte membranes showed that $(Na^+ + K^+)$-ATPase activity varied by 29.6% per 0.5 ml assay volume and 33.3% when expressed as per mg membrane-bound protein (data not shown). This variability was due to increases as well as decreases of enzyme activity over the 6-day period. On addition of 1% (w/v) saponin in the assay medium, the variability of $(Na^+ + K^+)$-ATPase over the 6-day period decreased to 6% (per 0.5 ml assay volume) and 20% (per mg membrane-bound protein) (Table II). In the absence of saponin $(Na^+ + K^+)$-ATPase activity was reduced from 0.506 to 0.405 μmol P_i/h per mg membrane-bound protein and from 0.177 to 0.154 μmol P_i/h per 0.5 ml assay volume (Table II).

TABLE II

$(Na^+ + K^+)$-ATPase VARIABILITY IN THE PRESENCE OF 1% (w/v) SAPONIN IN THE ASSAY MEDIUM

$(Na^+ + K^+)$-ATPase was assayed immediately after preparation and then kept at 4°C and reassayed on five occasions. To determine the effect of saponin on ATPase activity a control was included without saponin in the assay medium and measured together with the sixth estimation. Each value is the mean of triplicate samples with a C.V. of less than 5%.

A. Erythrocyte membranes prepared by hypotonic haemolysis and suspended in assay medium containing 1% (w/v) saponin.

Estimation (days)		$(Na^+ + K^+)$-ATPase activity	
		μmol P_i/h per mg membrane-bound protein	μmol P_i/h per 0.5 ml AV
1		0.668	0.167
2		0.683	0.164
3		0.449	0.175
4		0.432	0.162
5		0.522	0.188
6		0.506	0.177
	$\bar{x}$	0.543	0.172
	S.D.	0.108	0.010
	C.V. (%)	20	6
B. Control without 1% (w/v) saponin		0.405 ± 0.042	0.154 ± 0.008

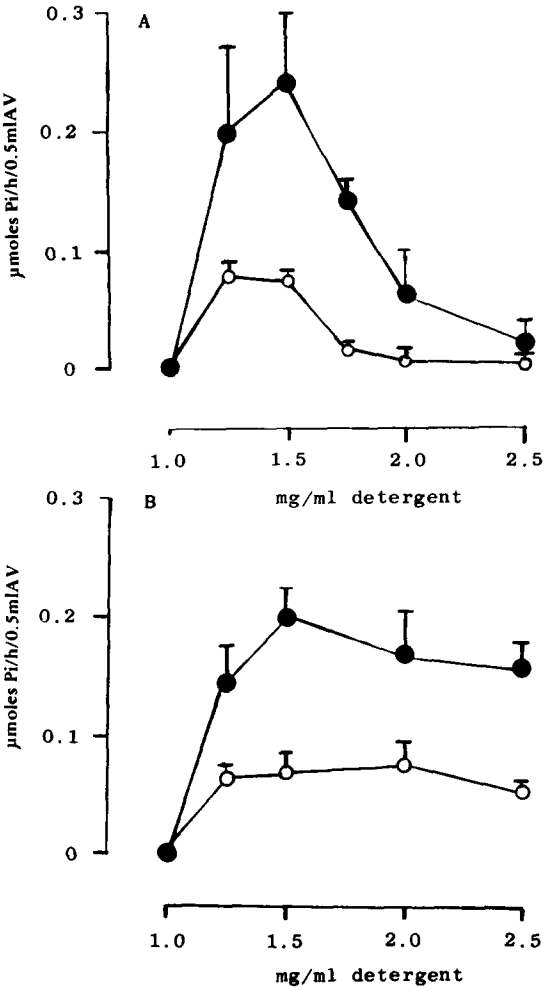

Fig. 1. Determination of optimal $(Na^+ + K^+)$-ATPase using serial concentrations of deoxycholate (A) and saponin (B); only data above 1.0 mg/ml are shown because haemolysis did not occur below this concentration. Serial concentrations of A and B were added to a 10% (v/v) erythrocyte suspension in solution II (see Methods). Four sources of blood from normal volunteers were assayed for ATPase activities in triplicate. The $(Na^+ + K^+)$-ATPase component was measured as the ouabain (10^{-4} M) inhibitable proportion of total ATPase. ATPase activity is expressed as phosphate (P_i) release per 0.5 ml assay volume (AV). Means ± S.E. ($n = 4$) are plotted. ●——●, total ATPase; ○——○, $(Na^+ + K^+)$-ATPase.

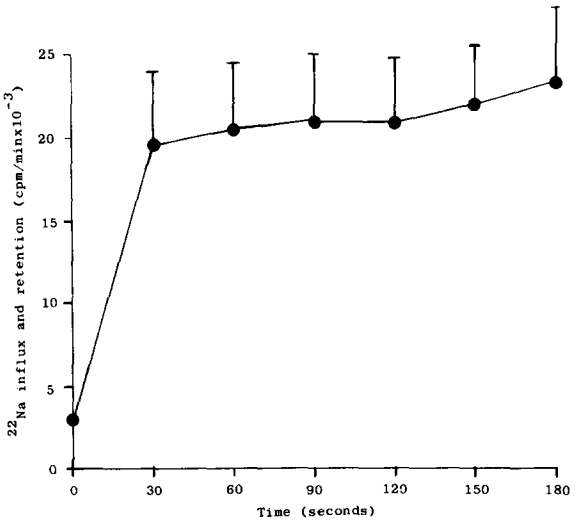

Fig. 2. Passive ^{22}Na influx into vesicles. 500 μl erythrocyte membrane vesicles (see Methods) were preincubated at 37°C in 500 μl of solution III. After 15 min ^{22}Na (5 μCi/ml) was added and 100-μl aliquots were taken at 30-s intervals. Each aliquot was filtered through a 0.45 μm filter and rapidly flushed with chilled 2×3 ml washes of solution III. The filters with the trapped membrane vesicles were place into plastic stoppered tubes and counted for either 10 min or 10000 counts (whichever was achieved first) on an LKB gamma counter. Samples were run against a suitable control to eliminate ^{22}Na which was trapped on the filters. A rapid influx of ^{22}Na is followed by a rapid equilibrium. Means+S.E. ($n=29$) are plotted.

Comparison of saponin and deoxycholate on ATPase activity

Since saponin treatment yielded an increased enzyme activity and reduced variability (Table II), we compared the effects of this non-ionic detergent with the ionic detergent sodium deoxycholate on red cell haemolysis and ATPase activity. Both detergents yielded maximum ATPase activities at a concentration of 1.5 mg/ml (Fig. 1). Deoxycholate released higher $(Na^+ + K^+)$-ATPase activity (0.165 ± 0.060 μmol P_i/h per 0.5 ml assay volume compared with 0.130 ± 0.025 P_i μmol/h per 0.5 ml assay volume) than saponin. Deoxycholate also exhibited a more rapid deactivation of enzyme acticity at higher haemolysing concentrations compared with saponin.

^{22}Na infux into erythrocyte vesticles

As a monitor of membrane permeability we investigated the passive (ouabain-uninhibited) ^{22}Na influx into erythrocyte vesicles. A rapid influx would indicate 'leaky' vesicles since sodium uptake into intact erythrocytes is known to be slow. After a rapid influx of ^{22}Na within 30 s, equilibrium was achieved and the intravesicular ^{22}Na remained constant (Fig. 2).

Comparison of deoxycholate, saponin and 10 imosM Tris-HCl on $(Na^+ + K^+)$-ATPase and vesicular 'leakiness'

Blood was drawn from four subjects and membranes prepared by haemolysis with either deoxycholate (1.5 mg/ml), saponin (1.5 mg/ml) (see method II) or 10 imosM Tris-HCl in a 10% cell suspension (see method I). $(Na^+ + K^+)$-ATPase and ^{22}Na influx were determined in all preparations. Both detergents yielded ATPase levels and ^{22}Na influx significantly ($P < 0.001$) greater than 10 imosM Tris-HCl (Table III). The rate of sodium influx when deoxycholate or saponin were used to haemolyse the erythrocytes was 2–3-times greater than when the haemolysis was achieved with 10 imosM Tris-HCl. $(Na^+ + K^+)$-ATPase activities were from 8- to 11-fold greater with detergent compared to hypotonic Tris.

TABLE III

COMPARISON OF HAEMOLYSIS WITH SODIUM DEOXYCHOLATE SAPONIN AND 10 mM TRIS-HCl ON ERYTHROCYTES, $(Na^+ + K^+)$-ATPase AND $^{22}Na^+$ INFLUX

Washed erythrocytes were haemolysed with either deoxycholate, saponin or 10 mM Tris-HCl for 60 min at 4°C with continual stirring (see Methods). Immediately after preparation the erythrocyte membrane vesicles were assayed for ATPase (37°C) and studied for ^{22}Na influx. Blood samples from four healthy volunteers were assayed in triplicate. Figures shown are means ±1 S.D. Significance was determined by the Student's paired *t*-test.

Haemolysing agent	$(Na^+ + K^+)$-ATPase (μmol P_i/h per 0.5 ml AV)	^{22}Na influx rate (cpm/min per 0.5 ml AV)
DOC (1.5 mg/ml)	0.165±0.060	1200±430
Saponin (1.5 mg/ml)	0.130±0.025	1500±300
Tris-HCl (10 mM)	0.015±0.016 [a]	470±133 [a]

[a] $P < 0.001$ when compared against deoxycholate and saponin values.

Reproducibility and expression of $(Na^+ + K^+)$-ATPase activity

Having established that 1.5 mg/ml deoxycholate yielded maximal ATPase, two important questions remained; first, whether blood obtained from the same subject on two different occasions would give reproducible $(Na^+ + K^+)$-ATPase activity, and secondly, whether this activity could be related to other indices. So far we had related ATPase activity to the amount of protein in the sample and to the assay volume; the former showed higher variability than the latter. Our studies with vesicle analysis on the Coulter counter and channelyzer suggested that ATPase activity could also be related to the number of vesicles present in the suspension. However, using deoxycholate under strictly standardized procedures ensured a consistent vesicle yield from a constant volume, and therefore the relationship to assay volume could be extended to its logical parameter of volume (ml or litre) of packed red cells.

In order to study $(Na^+ + K^+)$-ATPase reproducibility from preparation to preparation in the same subject we collected blood from four subjects on days 1 and 14 under identical conditions. Vesicles were prepared with 1.5 mg/ml deoxycholate and assayed immediately after preparation. Table IV shows the result, expressed as μmol P_i/h per 10^{10} vesicles, per ml of erythrocytes and per mg protein. As previously shown, assay volume extrapolated to a ml of erythrocytes remained the best index (Table II) of $(Na^+ + K^+)$-ATPase activity. With the introduction of deoxycholate and improvements of methodology this study showed the least mean difference (minimum variability) when ATPase activity was related to erythrocyte volume. In subjects 2 and 3 the ATPase activity was highly reproducible (Table IV). In comparison of the other two indices (protein and vesicles), vesicles showed better reproducibility with a mean difference of 25.9%.

Indicators of reproducibility

By studying and comparing various morphological parameters (see below) we were able to monitor ATPase reproducibility of vesicle preparations. An examination of erythrocyte membrane vesicle size-distribution graphs of subject 2 (Figs. 3 A and 3B, a typical example of a good reproducibility (Table IV), shows similar profiles with the same vesicle diameters between days 1 and 14. Protein concentration and vesicle counts of the two samples also exhibited little variation (less than 10%) as can be seen in Table V. $(Na^+ + K^+)$-ATPase from this subject when related to a ml of red cells showed activities from days 1 and 14 to agree within 9%; similar to the variation which occurred in the vesicle morphology. Blood from

TABLE IV

DAY TO DAY REPRODUCIBILITY OF $(Na^+ + K^+)$-ATPase ACTIVITY IN FOUR SUBJECTS

Erythrocyte membranes obtained from four volunteers on two separate occasions (day 1 and day 14) and assayed for $(Na^+ + K^+)$-ATPase activity after initial haemolysis with 1.5 mg/ml sodium deoxycholate. To determine the index which exhibits the least variability the three indices, litre erythrocytes, mg protein and number of vesicles were compared. Each datum is the mean of triplicates with less than 5% coefficient of variance.

Subject	μmol P_i/h per ml erythrocytes			μmol P_i/h per mg protein			μmol P_i/h per 10^{10} vesicles		
	Day 1	Day 14	% change	Day 1	Day 14	% change	Day 1	Day 14	%change
1	0.70	0.93	+32.8	0.26	0.60	+130.8	3.0	1.8	−40.0
2	0.90	0.98	+8.9	0.40	0.44	+10.0	1.6	1.9	+18.8
3	0.97	1.01	+4.1	0.39	0.63	+61.5	3.6	2.22	−38.9
4	0.79	0.63	−20.3	0.28	0.50	+78.6	3.0	1.7	−43.3
Mean of change			+8.4			+70.2			−25.9
S.D.			21.8			49.8			29.8
Range		−20.3 to +32.8			+10.0 to +130.8			−43.3 to +18.8	

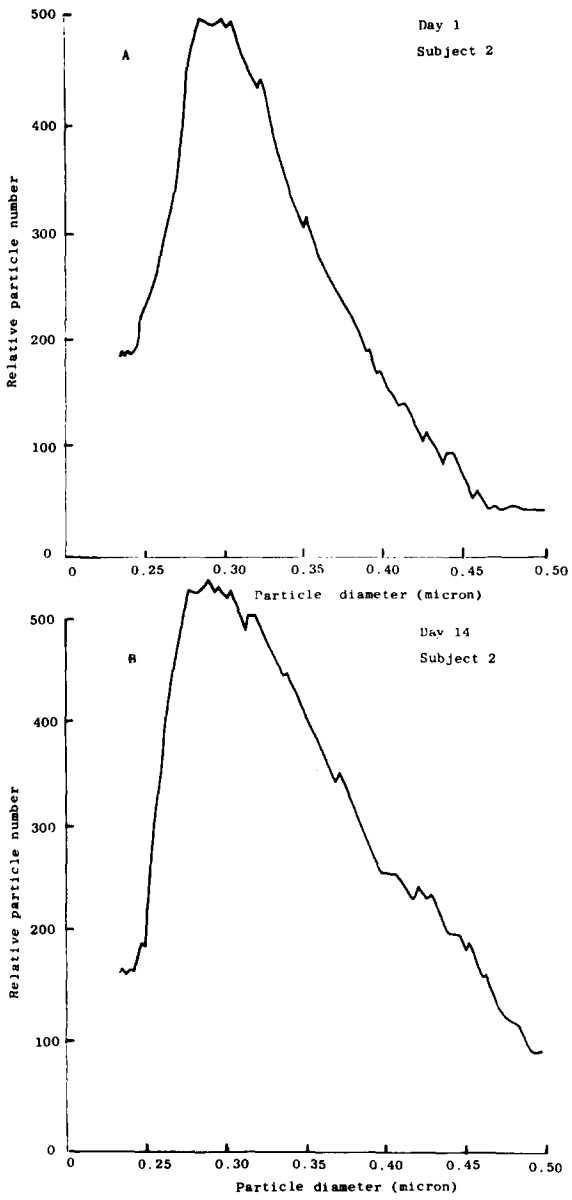

Fig. 3. Size-number curves drawn by a Coulter channelyzer graph recorder on day 1 (A) and day 14 (B) from vesicles on subject 2. The two profiles appear similar and $(Na^+ + K^+)$-ATPase activity agreed to within 18.8% when related to vesicle concentration (see Table IV). Mean particle diameter is 0.3 ± 0.2 μm (S.E.).

subject 4, also obtained on days 1 and 14, showed $(Na^+ + K^+)$-ATPase activity to vary by 20.3%. The vesicle count as well as the protein concentration of the membrane suspension showed a great variability from day 1 to day 14. The morphological features of erythrocyte vesicles from subject 4 varied and this was reflected by the loss of reproducibility in the $(Na^+ + K^+)$-ATPase activity. A similar scrutiny of the data from all the subjects studied in our laboratory has shown that morphological parameters are a good guide of vesicle preparation and of $(Na^+ + K^+)$-ATPase reproducibility. Morphologically similar erythrocyte preparations show: (1) Complete haemolysis of erythrocytes by deoxycholate. (2) Size-number graphs with the same mean vesicle diameter and similar profiles. (3) Protein concentration varying by less than 10%. (4) Vesicle number varying by less than 10%.

Inter-subject and inter-investigator variability

Exploratory experiments after an overnight fast and after breakfast showed that some of the variability in the ATPase activity of subjects 1 and 4

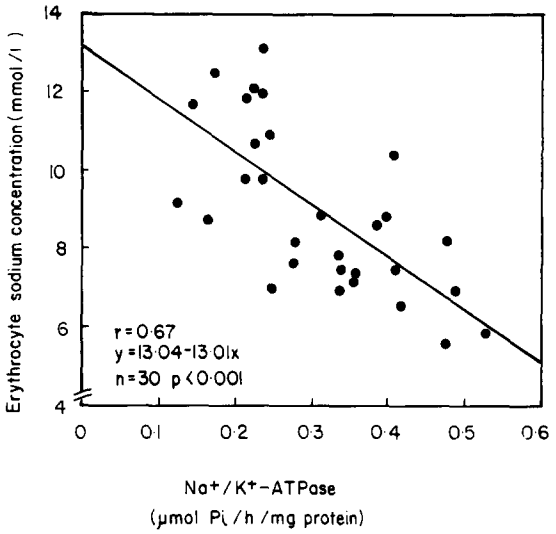

Fig. 4. Correlation between erythrocyte sodium concentration and $(Na^+ + K^+)$-ATPase activity obtained after the erythrocytes were haemolysed with sodium deoxycholate and vesicles were prepared (see Method II). Blood was obtained from three normal volunteers on four occasions after an overnight fast and 30 separate experiments were performed by two operators. Erythrocyte sodium concentration [25] and packed erythrocyte volume were determined in an aliquot from each blood sample, which was then used for the vesicle preparation. Vesicular protein concentrations were performed on the final vesicle suspension according to Hartree [21].

TABLE V

PROTEIN, VESICLE COUNT AND ATPase ANALYSES OF ERYTHROCYTE MEMBRANES FROM A SAMPLE EXHIBITING GOOD REPRODUCIBILITY (SUBJECT 2, TABLE IV) AND ANOTHER SAMPLE SHOWING POOR REPRODUCIBILITY (SUBJECT 4)

$(Na^+ + K^+)$-ATPase was assayed on erythrocyte membranes prepared on days 1 and 14. Protein was measured according to Hartree [21] and P_i released by $(Na^+ + K^+)$-ATPase activity was assayed by a modification of Lin and Morales [23]. The concentration of erythrocyte vesicles was determined by Coulter counter and channelyzer analysis. The change in each of the measured parameters from day 1 to 14 are expressed as % difference of day 1 and are shown in brackets. The poor reproducibility of $(Na^+ + K^+)$-ATPase activity between day 1 and day 14 in subject 4 is indicated by variable vesicle counts and protein concentrations.

Day of preparation	Subject	Protein (mg/ml assay volume)	Vesicles (10^9/0.5ml assay volume)	$(Na^+ + K^+)$-ATPase (μmol P_i/h per ml RBC)
1	2	0.201	5.05	0.90
14	2	0.198 (1.5%)	4.58 (9.3%)	0.98 (8.9%)
1	4	0.250	2.36	0.79
14	4	0.113 (54.8%)	3.28 (38.9%)	0.63 (20.3%)

(Table IV) was due to variabilities in the dietary intakes. In order to establish that the assay was reproducible on different days in a week in the blood from the same source, even when performed by two workers independently, blood was obtained from two subjects and erythrocyte vesicles prepared on days 1, 2, 7 and 8 after a 10 h fast on each occasion. Each 40 ml blood sample was divided into four 10-ml portions; two 10-ml portions going to each of the two operators for the preparation and ATPase assay of erythrocyte vesicles on two separate occasions on the same

TABLE VI

INTERSUBJECT AND INTEROPERATOR VARIABILITY IN ERYTHROCYTE $(Na+K^+)$-ATPase ACTIVITY

Blood was obtained from two normal subjects on the days after a 10 h overnight fast. Each 40 ml sample was divided into four 10-ml portions and two 10-ml aliquots were assayed, where possible by each of two operators on two separate occasions on the same day. On day 1 only one operator performed the assays to give $n=6$ instead of $n=8$. On day 7 one operator performed the assay once while the other performed the assay twice; this was repeated on day 8 to yield $n=6$. Day to day and interqperator variabilities are expressed as the mean ± S.D. The weekly variabilities are shown as the percent difference between days 1 and 2 and days 7 and 8.

	$(Na^+ + K^+)$-ATPase (μmol P_i/h)		
	per ml RBC	per mg protein	per 10^{10} vesicles
Subject 1			
Days 1 and 2	0.97±0.21	0.36±0.05	1.45±0.24
($n=6$)	C.V.(%) 21.6	13.9	16.6
Days 7 and 8	1.06±0.14	0.34±0.08	1.49±0.34
($n=6$)	C.V. (%) 13.2	23.5	22.8
Percent difference between days 1 & 2 and days 7 & 8	9.3	5.6	2.8
Subject 2			
Days 1 and 2	0.58±0.04	0.21±0.02	1.06±0.33
($n=6$)	C.V. (%) 6.9	10.0	31.1
Days 7 and 8	0.60±0.20	0.19±0.05	0.81±0.20
($n=6$)	C.V. (%) 33.3	27.8	25.1
Percent difference between days 1 & 2 and days 7 & 8	3.4	9.5	23.6

day. However, it was not always possible to perform the analysis twice and the number of experiments is given in Table VI. Whatever the index used, both operators achieved a C.V. of less than 24% in 12 experiments when assaying the blood from subject 1 and less than 34% in subject 2. The percent difference in the ATPase activity between days 1 and 2 and days 7 and 8 was the lowest when erythrocyte volume was used as an index of ATPase activity. Membrane-bound protein also emerged as an acceptable index but the vesicle count showed a low variability of the ATPase activity in subject 1 but a high and consistent variability in subject 2.

Validation of the method of erythrocyte vesicular ATPase activity

The data in Table VI demonstrate that a reproducible level of erythrocyte ATPase activity can be achieved from the same subject on different days under identical fasting and resting conditions with the method of erythrocyte vesicle preparation described here. We had to validate these results by showing a correlation between the data on vesicles and the data on intact erythrocytes. Blood was obtained on four occasions from three normal subjects (two male and one female) after an overnight fast. A 2-ml aliquot from each sample was set aside for the determination of intracellular sodium concentration by a method described previously [25] and for packed cell volume. The remainder of the blood on each occasion was used for the preparation of vesicles (see Methods). As would be expected a significant ($P < 0.001$) negative correlation ($r = 0.67$) was obtained between the erythrocyte sodium concentration and $(Na^+ + K^+)$-ATPase activity per mg protein in erythrocyte vesicles (Fig. 4). Similarly, a significant ($P < 0.001$) negative correlation ($r = 0.61$) was observed when the ATPase activity was expressed per ml of erythrocytes (not shown).

Discussion

High and reproducible levels of $(Na^+ + K^+)$-ATPase can be obtained from erythrocytes with a strictly standardized protocol of vesicle preparation. The use of deoxycholate as a haemolysing agent ensured complete haemolysis and an increased permeability of erythrocyte membranes. Both these factors contributed to a reproducible yield of $(Na^+ + K^+)$-ATPase from preparation to preparation. Various workers [5, 15–19, 25–27] have suggested that detergents increase membrane permeability to substrates, inhibitors and activators thereby producing increased yields of $(Na^+ + K^+)$-ATPase. Our experiments support this concept because vesicles prepared in deoxycholate and saponin allow a rapid influx of ^{22}Na; both the $(Na^+ + K^+)$-ATPase and ^{22}Na are signficantly greater (table III) than in membranes prepared by low osmolarity (hypotonic) haemolysis.

Bramley et al. [5] studying the effects of detergents and sonication techniques on erythrocyte membranes prepared initially with moderate osmolarities showed that detergents and sonication 'unmasked' latent ATPase. This was due to an increased permeability of membranes to the substrate [γ-^{32}P]-ATP. These workers suggested that complete haemolysis and increased permeability caused by detergents or low osmolar solutions produced more demonstrable ATPase. Our experiments showed that if erythrocyte membranes were prepared with hypotonic haemolysis and frozen, saponin increased the ATPase activity (data not shown) and membranes kept at 4°C also exhibited reduced variability; but if membranes were prepared with deoxycholate any further detergent treatment decreased ATPase activity. In fact such treatment solubilized membrane-bound protein and reduced the concentration of vesicles in the membrane preparations. Contrary to Bramley et al. [5] we found membranes were not fully permeable when prepared by low osmolarity solutions (10 imosM Tris-HCl), as shown by reduced ^{22}Na permeability and lower $(Na^+ + K^+)$-ATPase activity. Both the permeability and enzyme activity were significantly improved by treatment with detergents. Our results suggest that detergents may open up new channels in membranes through which all substrates, activators and inhibitors can gain access to ATPase sites. Detergent haemolysis is thus not only important because it releases maximal $(Na^+ + K^+)$-ATPase but also because it alleviates any problems arising from membrane 'sidedness' [28]. In other words, the presence of varying proportions of 'right-side-out' and 'inside-out' vesicles is no longer relevant, when the vesicles

are rendered fully permeable by treatment with deoxycholate. Some workers [29] have even attempted to isolate 'inside-out' vesicles as an alternative to making the vesicles 'leaky'. The initial rapid influx of ^{22}Na in these studies clearly illustrates the several hundred fold increased permeability of vesicle membranes compared to sodium equilibration in intact erythrocytes which occurs after 2–3 h.

Erythrocyte membranes form vesicles after treatment with detergents and homogenization [5, 30]. Deoxycholate has been reported to be more damaging to erythrocyte membranes than other detergents [30]. Our findings agree with these reports and show that if deoxycholate is used in excess of 1.5 mg/ml solubilization and deactivation of enzyme activity is more marked compared with saponin. At the optimal concentration of 1.5 mg/ml deoxycholate uniform and highly permeable vesicles are produced. This not only yields high levels of $(Na^+ + K^+)$-ATPase but also a size-number profile on channelyzer analysis which acts as an indicator to membrane vesicle reproducibility between preparations. Thus by monitoring differences in morphological details (size-number graphs, vesicle and protein concentrations) any changes in $(Na^+ + K^+)$-ATPase between preparations from the same subject due to preparatory artifacts may be eliminated. Erythrocyte and assay volumes were found to be the most reliable indices of ATPase activity. In addition the vesicle count of each sample can provide an index as well as a monitor for preparatory variations. However, as compared with our previously published results [25], erythrocyte ATPase activity related to vesicle count is 10-times greater in this study. This is entirely due to the fact that we discarded non-vesicular particle detection with a mean diameter of less than 0.25 μm. This improved the monitoring potential of vesicle size graphs (see Methods). The resulting difference in vesicle count, and thereby in the ATPase activity related to it, does not affect the actual ATPase activity, which is the same when related to erythrocyte volume and vesicular protein, and about 10-times greater in all subjects when related to vesicle count.

There are numerous reasons for variable ATPase activities between preparations and from assay to assay. ATPase components are located on the inner membrane surface [31], and the cell needs to be rendered leaky to ATP for the ATPase activity to be revealed. Membranes prepared by hypotonic lysis in EDTA were found not to be completely haemoglobin-free and 'leaky' to ATPase as suggested by other workers [16, 32], and yielded variable ATPase activity from preparation to preparation. During our preliminary studies it was found that even a slight deviation from any procedural detail (i.e. mixing, washing, centrifugation, etc.) produced variations in both the degree of haemolysis and in the ATPase yield. Strict adherence to this protocol was largely responsible for reproducible results of $(Na^+ + K^+)$ ATPase levels from assay to assay. However, even meticulous attention to detail and the use of deoxycholate may not always produce the exact $(Na^+ + K^+)$-ATPase levels on two different days in erythrocytes from the same subject (subjects 1 and 4 in Table IV). Experiments with erythrocyte $(Na^+ + K^+)$-ATPase levels before and after a meal (unpublished data) have demonstrated that erythrocyte ATPase is not static as we had presumed; a presumption based on the fact that erythrocytes have no organelles and have a long life span. Erythrocyte $(Na^+ + K^+)$-ATPase increases in response to energy intake.

Day to day and assay to assay ATPase activity was rendered reproducible when blood samples were collected under identical fasting and resting conditions from the same subjects (Table VI). This reproducibility was not disturbed even when two investigators prepared erythrocyte vesicles and performed the ATPase assay. A highly significant ($P < 0.001$) negative correlation between erythrocyte sodium concentration and erythrocyte vesicle ATPase activity (Fig. 4) further supports our contention that our method of vesicle preparation with deoxycholate achieves maximal yield of ATPase components from intact erythrocytes.

In conclusion, the method described here will yield maximal and reliable levels of $(Na^+ + K^+)$-ATPase from assay to assay: day to day variations may be of biological nature but can also be reduced if blood samples are collected under identical conditions and if all the steps of preparation are strictly adhered to. Erythrocytes may prove to be a good model for energy intake studies, and this method should be useful in such experiments.

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