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South-east Asian ovalocytic (SAO) erythrocytes have a cold sensitive cation leak: implications for in vitro studies on stored SAO red cells

Lesley J. Bruce [a], Susan M. Ring [a], Kay Ridgwell [a], David M. Reardon [b], Carol A. Seymour [c], Heidi M. Van Dort [d], Philip S. Low [d], Michael J.A. Tanner [a,*]

[a] *Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, UK*
[b] *Department of Haematology, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK*
[c] *Division of Cardiological Sciences (Metabolic Medicine), St George's Hospital Medical School, London SW17 0RE, UK*
[d] *Department of Chemistry, Purdue University, West Lafayette, IN 47907-1393, USA*

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Abstract

South-east Asian ovalocytosis (SAO) results from the heterozygous presence of an abnormal band 3, which causes several alterations in the properties of the erythrocytes. Although earlier studies suggested that SAO erythrocytes are refractory to invasion in vitro by the malarial parasite *Plasmodium falciparum,* a more recent study showed that fresh SAO cells were invaded by the parasites, but became resistant to invasion on storage because intracellular ATP was depleted more rapidly than normal. Here we show that SAO red cells are much more leaky to sodium and potassium than normal red cells when stored in the cold. This leak was much less marked when the cells were stored at 25 or 37°C. Incubation for 3.5 h at 37°C of cold-stored SAO red cells did not restore sodium and potassium to normal levels, probably because the depleted ATP level in cold-stored SAO red cells is further reduced with incubation at 37°C. The increased leakiness of SAO red cells is non-specific and extends to calcium ions, taurine, mannitol and sucrose. These results suggest that SAO red cells undergo a structural change on cooling. Since many of the reports describing altered properties of SAO red cells have used cells which have been stored in the cold, these results need re-evaluation using never-chilled SAO red cells to assess whether the cells have the same abnormal properties under in vivo conditions.

Keywords: Band 3; Ovalocytosis; Malaria parasite; Cation leak; Anion exchanger (AE1)

1. Introduction

South-east Asian ovalocytosis (SAO) is a hereditary condition characterised by elliptocytic red cells with slit-like depressions. The condition is also referred to as hereditary ovalocytosis or stomatocytic elliptocytosis. SAO has been shown to result from the heterozygous presence of an abnormal red cell anion exchanger (band 3). SAO band 3 has a deletion of amino acids 400–408 and is linked to the relatively common band 3 Memphis polymorphism, $Lys_{56} \rightarrow Glu$ [1–3]. The protein is inactive in anion transport and does not bind some anion transport inhibitors [4–7].

SAO occurs with relatively high frequency in some south-east Asian and Melanesian populations, including Malaysia and Papua New Guinea [8,9], and has also been observed in a family of Indian descent

* Corresponding author. Fax: +44 (117) 928-8274; E-mail: m.tanner@bris.ac.uk

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and an African-American family [3,10]. The prevalence of SAO in areas where malaria is endemic led to suggestions that the condition might confer some protection against malaria [9,11]. Recent work suggests that SAO children are protected against cerebral malaria, the potentially fatal complication of the disease [12]. Studies in vitro suggested that SAO erythrocytes are resistant to invasion by *Plasmodium falciparum* and *Plasmodium knowlesi* [13,14]. SAO red cells are also much more rigid than normal red cells, and their resistance to malaria invasion was attributed to the altered membrane mechanical properties of the cells [15,16].

However, the in vitro results suggesting that SAO red cells are refractory to parasite invasion are at variance with in vivo studies on adult populations in Papua New Guinea [17]. Cattani et al. [17] showed that although the prevalence of *P. falciparum* and *Plasmodium vivax* infections was slightly lower in SAO than normal individuals, the same degree of parasitemia was observed in the parasitised individuals of the two groups, suggesting that once infected, the red cells of SAO individuals have a susceptibility to parasite invasion in vivo which is similar to that of normal individuals [17]. Support for this view has come from recent in vitro data which demonstrate that SAO red cells show substantial invasion by *P. falciparum* in culture (at least 55% that of normal cells) when the SAO cells were studied 5 h from when the blood was drawn [18]. The extent of invasion was found to fall off much more rapidly for the SAO red cells than normal cells on storage in the cold. This was not accompanied by any change in the rigidity of the SAO cells. However, the intracellular ATP content of the SAO red cells fell dramatically on storage, and was markedly lower than the normal cells just 5 h. after the blood was drawn [18]. Incubation of the SAO red cells in a metabolite regenerating medium led to increases in intracellular ATP and susceptibility to invasion by *P. falciparum*, but to a lesser extent than normal cells. Dluzewski et al. [18] suggest that the decrease in intracellular ATP concentration is responsible for the rapid decline in invasion of SAO red cells on storage, and conclude that the earlier observations that SAO red cells were refractory to invasion probably reflected long times of storage between collecting the blood samples and carrying out the invasion assays [13,14].

In this study we showed that freshly drawn SAO red cells were slightly more leaky to sodium and potassium ions than control red cells at 25°C and above, and became markedly more leaky to monovalent cations when maintained at 0°C. Incubation for 3.5 h at 37°C of cold-stored SAO red cells did not restore sodium and potassium to normal levels, partly because SAO red cells stored at 0°C were rapidly depleted of ATP and incubation at 37°C only depleted these cells further of their ATP. The loss of ATP as well as the abnormal cation content of SAO red cells stored in the cold probably give rise to their resistance to invasion by malarial parasites in vitro. SAO red cells were also shown to be noticeably more leaky to taurine, sucrose and mannitol than normal red cells.

2. Experimental

2.1. Methods

SAO blood samples were obtained from Mauritian twins (NG and SG), one of whom (NG) has been described [3,18,19] and a Filipino individual (Z) [6]. Examination of the blood was done in two laboratories, and the assays were carried out under slightly different conditions. SAO (Z): Blood was taken by venipuncture into acid-citrate-dextrose (ACD) anticoagulant containing 5 mM EGTA to chelate the plasma Ca^{2+}. SAO (NG and SG): Blood was taken by venipuncture into 5 mM EGTA and 10 mM glucose. The cells were assayed immediately and then stored either at 37°C or on wet ice at 0°C. Control red cells from normal adults were drawn at the same time and kept under the same conditions.

2.2. Sodium and potassium assays

SAO (Z): Blood was centrifuged immediately after being drawn into anticoagulant. The plasma and buffy coat were removed and the red cells resuspended at 15% haematocrit in a modified Krebs buffer (120 mM NaCl, 4.8 mM KCl, 5 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM HEPES, 2 mM EGTA, 10 mM glucose, pH 7.4), pre-equilibrated at 0, 25 or 37°C, with or without the sodium/potassium pump inhibitor, ouabain (0.1 mM), and the Na^+,K^+,Cl^-

co-transport inhibitor, bumetanide (0.1 mM). A sample of each red cell suspension was counted and the sodium and potassium content analysed by atomic absorption spectrometry.

SAO (NG and SG): The blood was kept as whole blood. No transport inhibitors were added. All aliquots were taken using a positive displacement pipette. The packed cell volume (PCV) of each sample was measured and the sodium and potassium content of the samples were analysed by flame photometry.

The cells were incubated at the appropriate temperature and sampled at various times over a period of 24 h. Samples were taken in triplicate and centrifuged through dibutyl phthalate. The cell pellet was washed twice with 100 mM $MgCl_2$, lysed with water and the proteins precipitated with perchloric acid. The supernatant or plasma and the cell lysate were analysed for sodium and potassium content using a flame photometer or atomic absorption spectrometer. To estimate the cation concentrations in the serum of freshly drawn blood, blood was drawn without anticoagulant, centrifuged immediately, and serum cation concentrations were measured using a Hitachi 717 Automatic Chemical Analyser.

2.3. Red cell lysis

Haemolysis tests were performed using the standard hemiglobincyanide method [20].

2.4. Measurement of Na^+,K^+-ATPase activity and leak fluxes

Red cells were either stored on wet ice at 0°C or examined immediately after being drawn. Except for the freshly drawn samples, red cells were incubated in modified Krebs buffer (as above) for 2 h at 37°C, before carrying out the assay. The uptake of $^{86}Rb^+$ (used as a tracer for K^+) into the cells was measured as described by Stewart [21]. Briefly, SAO and control red cells were washed three times in buffered saline (150 mM NaCl, 15 mM MOPS, 2 mM EGTA, 5 mM glucose, pH 7.4), then samples of the cells were resuspended at a 6% haematocrit in buffered saline alone, and in buffered saline with 0.1 mM ouabain, and in buffered saline with 0.1 mM ouabain and 0.1 mM bumetanide. KCl (final concentration 12.2 mM) containing tracer $^{86}Rb^+$ was added and the influx of radioactivity measured at 37°C over 30 min. The ouabain sensitive potassium influx was calculated by subtracting the influx in the presence of ouabain from the influx in the absence of inhibitors. The bumetanide sensitive influx was calculated by subtracting the influx in the presence of ouabain and bumetanide from the influx in the presence of ouabain alone. The non-specific leak was defined as the influx in the presence of both inhibitors.

2.5. Calcium uptake

Red cells, stored at 0°C for 3 days, were suspended in modified Krebs buffer (as above) and incubated for 2 h at 37°C. The cells were washed and resuspended at a 6% haematocrit in 145 mM NaCl, 5 mM $MgSO_4$, 25 mM HEPES, 0.5 mM vanadate (a Ca^{2+}-ATPase inhibitor), 0.1 mM quinidine (a Gardos channel inhibitor) and 5 mM glucose, pH 7.4. The suspension was incubated for 15 min at 37°C to allow binding of the inhibitors. $CaCl_2$, containing tracer [^{45}Ca]$CaCl_2$, was added to a final concentration of 1.5 mM and the suspensions were incubated at 25°C for 18 h, during which samples were taken in triplicate. The cell pellet was washed twice with ice-cold 0.15 mM NaCl, 0.5 mM vanadate, 0.1 mM quinidine, 1 mM EGTA and the [^{45}Ca] calcium uptake into the cells was determined by scintillation counting.

2.6. Taurine, sucrose and mannitol uptake

Red cells stored at 0°C for 4 days were washed and resuspended at a 10% haematocrit in modified Krebs buffer containing either 0.1 mM taurine, 0.1 mM sucrose or 0.1 mM mannitol and trace amounts of the ^{14}C-labelled compound. The cells were incubated at 0°C and samples were taken in triplicate over a period of 69 h [^{14}C]taurine or [^{14}C]sucrose or [^{14}C]mannitol uptake into the cells was measured by scintillation counting.

2.7. Adenine nucleotide levels

Fresh blood samples [SAO (Z) and control] were mixed with an equal volume of 3.7 M perchloric

acid. The supernatant was neutralised with potassium hydroxide and the adenine nucleotide levels assayed by standard methods [22].

Fresh blood samples [SAO (NG and SG) and control], and red cells washed with 0.15 M NaCl, were treated as above and the adenine nucleotide levels assayed by standard methods [22].

3. Results

3.1. Red cell and serum sodium and potassium concentrations in freshly drawn SAO blood

The concentrations of sodium and potassium ions in SAO and control red cells and serum were measured within 5 min of the blood samples being drawn (Table 1). The intracellular sodium concentration, and serum sodium and potassium ion concentrations of the SAO samples were not very different to those of normal controls. However, the intracellular potassium concentration of the red cells of all the SAO donors was found to be slightly lower than in control red cells measured under the same conditions (Table 1). The statistical significance of this trend will have to await the analysis of additional freshly drawn SAO blood samples.

3.2. Temperature dependence of the leakiness of SAO red cells to sodium and potassium ions

We used freshly drawn SAO and control red cells to measure the time course of the leak of sodium and potassium ions at different temperatures. The results obtained on incubation of freshly drawn SAO (NG and SG) cells and normal red cells at 0 and 37°C are shown in Fig. 1. This experiment was done using whole blood samples; at each time point red cell lysis was measured and found to be less than 2%. Incubation at 0°C resulted in the SAO red cells becoming profoundly leaky to sodium and potassium compared to control red cells, whereas when incubated at 37°C SAO (NG and SG) red cells lost potassium ions and gained sodium ions at a similar rate to control red cells (Fig. 1). Another experiment, using freshly drawn SAO (NG and SG) and control red cells, was done on a separate occasion with incubations at 25, 37 and 0°C either in the presence of ouabain and bumetanide or without inhibitors. Very similar results were obtained, with SAO (NG and SG) cells showing the same marked loss of potassium ions and gain in sodium ions at 0°C compared to normal red cells, and either a similar or slightly higher loss of potassium ions and gain in sodium ions compared with control cells at 25°C and 37°C, irrespective of whether ouabain and bumetanide were present (data not shown).

In a separate experiment freshly drawn SAO (Z) cells and normal red cells were incubated at 0°C, and 37°C in the presence and absence of ouabain and bumetanide. SAO (Z) red cells behaved like SAO (NG and SG) red cells and were also found to be profoundly leaky to both sodium and potassium compared to control cells when incubated at 0°C (Fig. 2a–c), but only slightly more leaky than control cells when incubated at 37°C, whether the inhibitors were present or not (Fig. 2d–f).

These results show that while SAO red cells are slightly more leaky to potassium and sodium ions than normal cells at 25°C and above, they are grossly leaky to these cations at 0°C compared with normal cells.

Table 1
Sodium and potassium ion concentrations in fresh SAO blood samples

Sample	Red cells (mmol (l cells)$^{-1}$)		Serum (mM)	
	Sodium	Potassium	Sodium	Potassium
Control	8 ± 0.8	90 ± 2.4	139	4
SAO (Z)	8 ± 1.1	82 ± 2.4	143	4
Control	7 ± 0	110 ± 1.0	146 ± 0.1	4 ± 0.01
SAO (NG)	n.t.	95 ± 0.7	147 ± 0.1	4 ± 0.04
SAO (SG)	n.t.	95 ± 1.7	146 ± 0.1	3.5 ± 0.02

The two sets of SAO samples should only be compared with the adjacent control samples. These were examined in the same laboratory on the same day. Where the standard deviation is shown the result is the mean of three replicates. n.t., not tested.

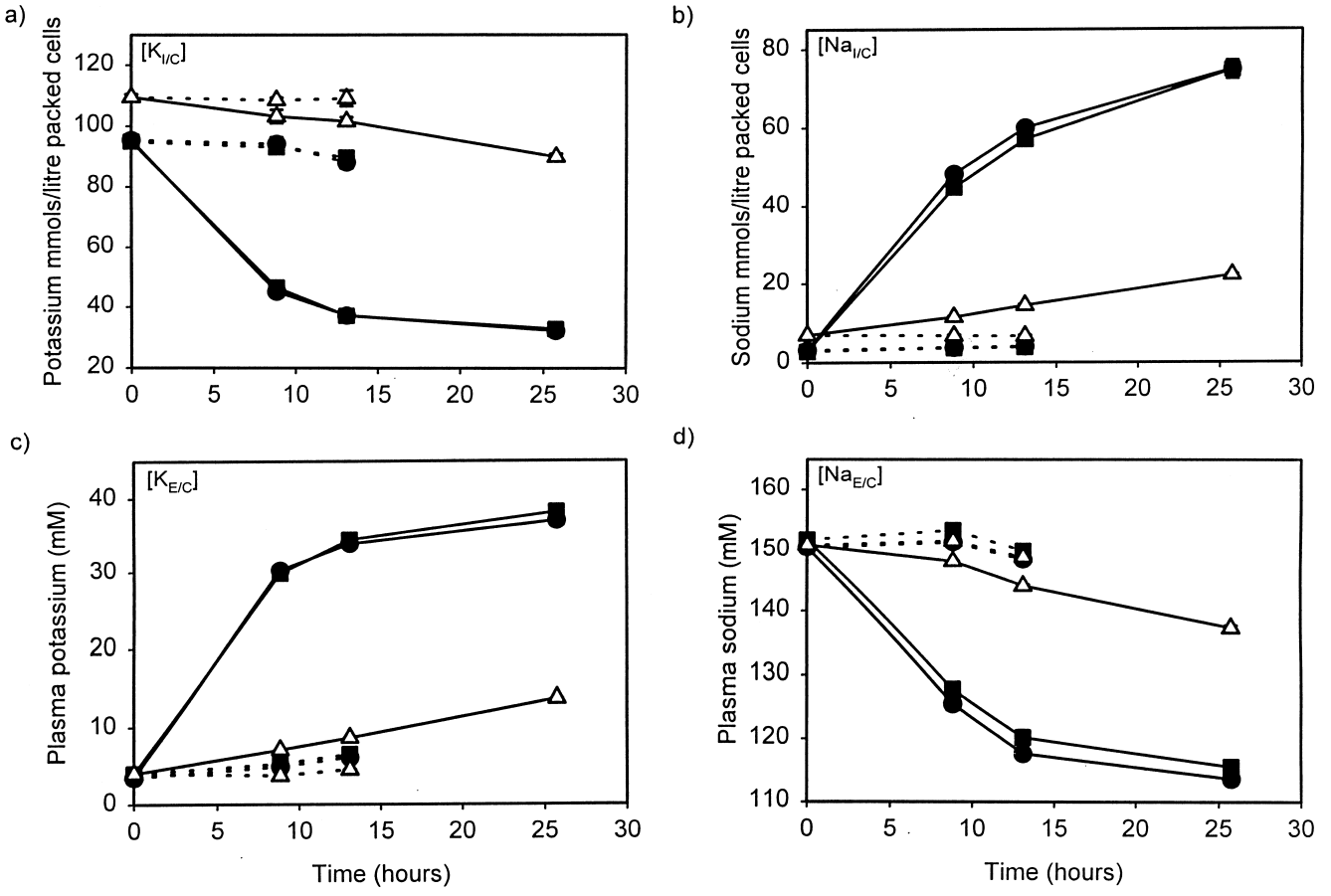

Fig. 1. (a, b) Red cell sodium and potassium ion concentrations in fresh SAO (NG and SG) and control samples, incubated as whole blood and sampled over 24 h (as described in Section 2). (c, d) The sodium and potassium ion concentrations of the plasma. SAO (NG) samples (■); SAO (SG) samples (●); control samples (Δ). (a) Intracellular K^+ concentration [$K_{I/C}$]. (b) Intracellular Na^+ concentration [$Na_{I/C}$]. (c) K^+ concentration of plasma [$K_{E/C}$]. (d) Na^+ concentration of plasma [$Na_{E/C}$]. Continuous lines: samples incubated on wet ice at 0°C; dashed lines: samples incubated at 37°C.

3.3. *The permeability of SAO red cells to other ions and molecules*

Since the SAO cells were leaky for both sodium and potassium ions in the cold, we examined the permeability of SAO cells to calcium ions and other molecules to gain an indication of the specificity of the leak pathway. The uptake at 25°C of calcium ions into SAO (NG and SG) and control red cells that had been stored at 0°C for 3 days was found to

Table 2
Uptake of calcium and small molecules into SAO and control cells

Sample	Calcium μmol (l cells)$^{-1}$ h^{-1}	Sample	Taurine μmol (l cells)$^{-1}$ h^{-1}	Mannitol μmol (l cells)$^{-1}$ h^{-1}	Sucrose μmol (l cells)$^{-1}$ h^{-1}
SAO (NG)	4.4 ± 0.1	SAO (Z)	0.3 ± 0.008	0.5 ± 0.02	0.07 ± 0.004
SAO (SG)	4.7 ± 0.4	Control	0.04 ± 0.003	0.1 ± 0.004	0.04 ± 0.006
Control 1	1.0 ± 0.1				
Control 2	1.4 ± 0.3				

The standard deviation shown is the deviation from the mean of three replicates.

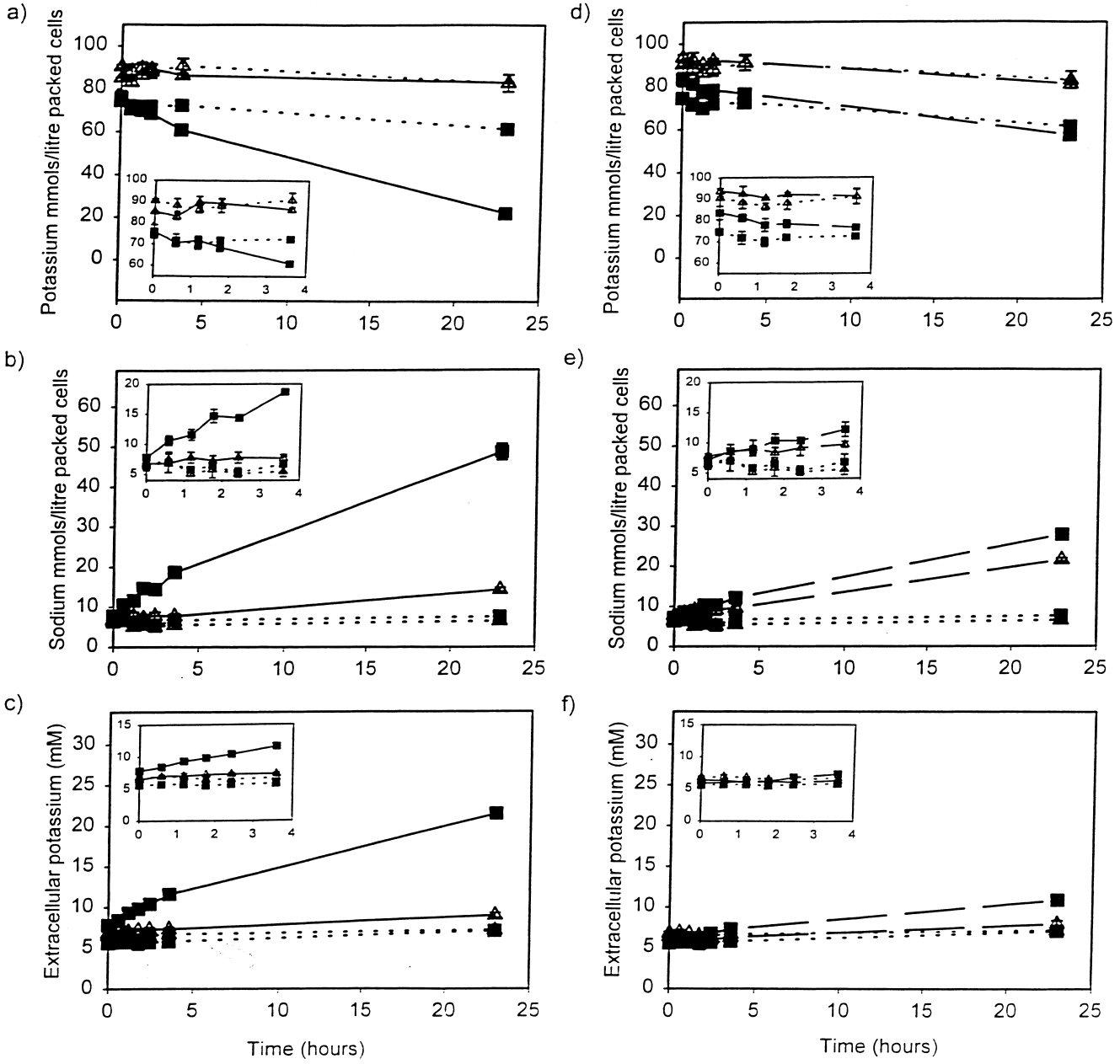

Fig. 2. (a, b, d, e) Sodium and potassium ion concentrations in fresh SAO (Z) and control red cell samples, incubated in modified Krebs buffer and sampled over 24 h (as described in Section 2). (c, f) The extracellular potassium ion concentration. SAO (Z) samples (■); control samples (Δ). (a, d) Intracellular K^+ concentration. (b, e) Intracellular Na^+ concentration. (c, f) K^+ concentration of plasma. Continuous lines: samples incubated on wet ice at 0°C; dashed lines: samples incubated at 37°C; broken lines: samples incubated at 37°C in the presence of 0.1 mM ouabain and 0.1 mM bumetanide. The inset boxes show an expanded view of the measurements over the first 4 h.

be about four times faster in SAO cells than in control red cells (Table 2). The uptake of taurine, sucrose and mannitol into SAO (Z) and control red cells that had been stored at 0°C for 4 days was also measured. Influx was minimal in control cells and noticeably greater in SAO (Z) red cells (Table 2). Similar results were obtained in three separate experiments to measure taurine uptake, and in two separate experiments to measure sucrose and mannitol uptake. The anion transport inhibitor DIDS had

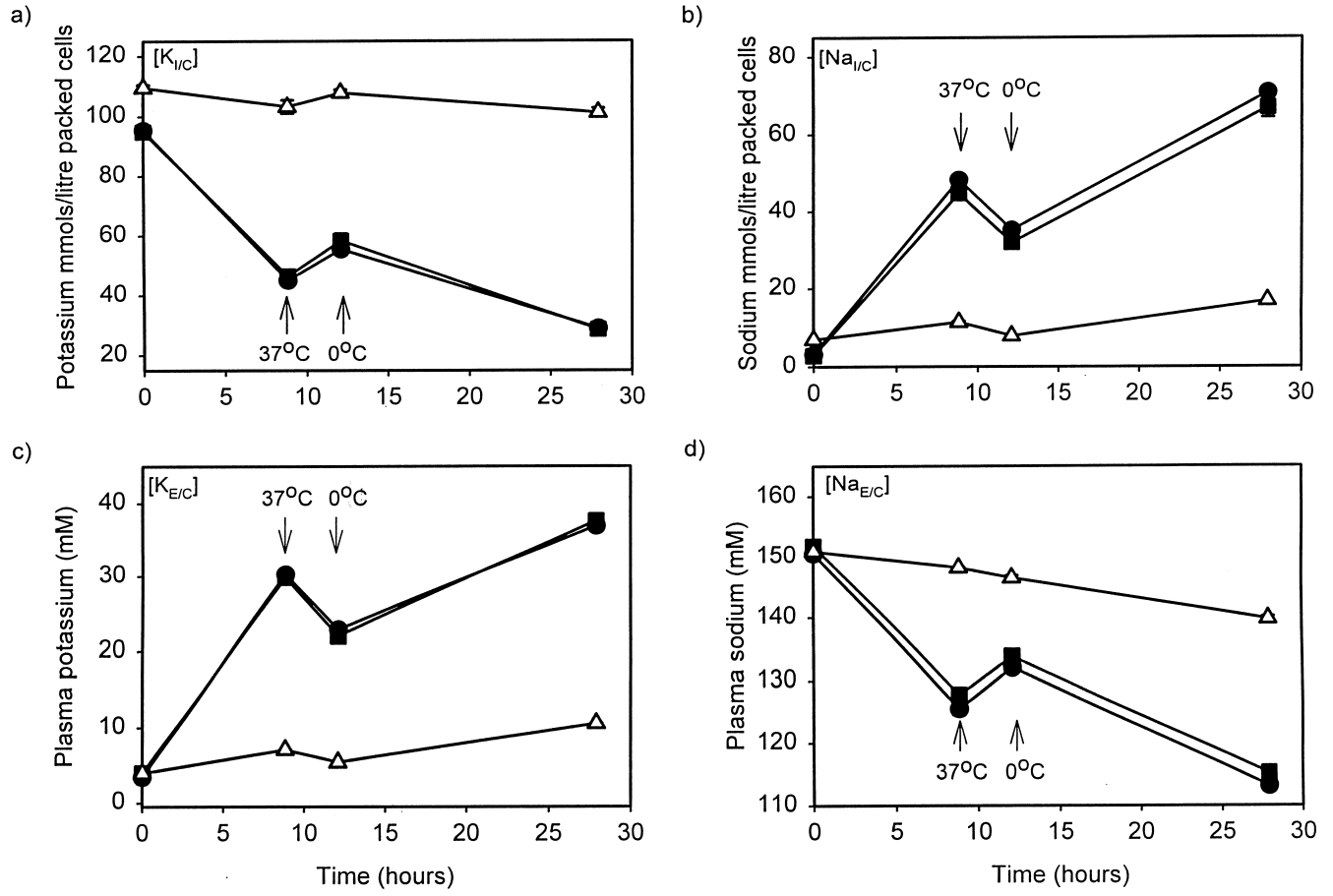

Fig. 3. (a, b) Changes in sodium and potassium ion concentrations measured in fresh SAO (NG and SG) and control red cell samples, incubated as whole blood for 8.5 h at 0°C, then 3.5 h at 37°C and then 16 h at 0°C (as described in Section 2). (c, d) The sodium and potassium ion concentrations in the plasma. SAO (NG) samples (■); SAO (SG) samples (●); control samples (Δ). (a) Intracellular K^+ concentration [$K_{I/C}$]. (b) Intracellular Na^+ concentration [$Na_{I/C}$]. (c) K^+ concentration of plasma [$K_{E/C}$]. (d) Na^+ concentration of plasma [$Na_{E/C}$]. Alterations in temperature are marked with an arrow.

no effect on taurine influx into the SAO or normal red cells.

3.4. Sodium pump activity and potassium leak in SAO red cells

The non-specific leak to potassium ions, the activity of the Na^+,K^+,Cl^- co-transporter and the activity of the Na^+,K^+-ATPase were measured at 37°C as described by Stewart [21]. The experiment was carried out on two different occasions using SAO (NG and SG) and control red cells, using never-chilled blood within 30 min of the blood being drawn or blood stored for 2 days at 0°C. The experiment was also carried out using SAO (Z) and control red cells after storage for 3 days at 0°C.

The Na^+,K^+-ATPase in freshly drawn SAO (NG and SG) red cells was about 8 times more active than normal controls, while the non-specific potassium leak in SAO (NG and SG) red cells was slightly greater than control red cells (Table 3). In cells that had been stored for 2 days at 0°C the activity of the Na^+,K^+-ATPase in SAO (SG) red cells was about 3.5 times that of normal red cells and the non-specific leak in SAO (NG and SG) red cells was about 4 and 6 times that of the controls respectively (Table 3). This reduction (compared to freshly drawn SAO cells) in activity of the Na^+,K^+-ATPase in SAO

Table 3
Sodium and potassium ion transport in SAO and control red cells[a]

Sample	Cells stored at 0°C for		Cells kept at 25°C and assayed within 30 min
	3 days	2 days	
Ouabain sensitive component			
Na^+K^+-ATPase (normal range: 0.9–2.0 mmol (l cells)$^{-1}$ h^{-1})			
Control	–	0.94	1.18
Control	–	0.71	1.36
SAO (NG)	–	–	9.94
SAO (SG)	–	3.10	10.63
Control	1.32	–	–
SAO (Z)	3.68	–	–
Bumetanide sensitive component			
$Na^+K^+Cl^-$ co-transporter (normal range: 0–0.8 mmol (l cells)$^{-1}$ h^{-1})			
Control	–	0.87	0.31
Control	–	0.60	0.29
SAO (NG)	–	–	0.30
SAO (SG)	–	0.0	0.30
Control	0.36	–	–
SAO (Z)	0.56	–	–
Ouabain and bumetanide insensitive component			
Non specific leak (normal range: 0.06–0.10 mmol (l cells)$^{-1}$ h^{-1})			
Control	–	0.13	0.19
Control	–	0.13	0.20
SAO (NG)	–	0.53	0.32
SAO (SG)	–	0.79	0.28
Control	0.23	–	–
SAO (Z)	0.71	–	–

[a]Measured with $^{86}Rb^+$ tracer as described in Section 2. Fluxes are expressed as mmol (l cells)$^{-1}$ h^{-1}.

(NG and SG) red cells after storage for 2 days probably results from the depletion of the ATP substrate in these cells (see below and [18]).

The non-specific potassium leak in SAO (Z) red cells was three times greater than the normal control (Table 3). It should be noted that the potassium leak in all the SAO red cells stored at 4°C was noticeably higher than the potassium leak in the freshly drawn SAO red cells which were never chilled. This is consistent with the experiments discussed above which show that the cation leak is increased on storage in the cold.

It was reported previously by Reardon et al. [19] that SAO (NG) had compensated haemolytic anaemia (peripheral blood reticulocyte count, 3.6% of red cells), which might contribute to the elevated Na^+,K^+-ATPase activity in the freshly drawn blood sample from SAO (NG) and SAO (SG). We examined the peripheral blood reticulocyte count in a sample from SAO (Z) and found that it was normal (0.3% of red cells). Although, it was not possible to carry out an experiment using freshly drawn blood from SAO (Z), measurement of Na^+,K^+-ATPase activity and non-specific leak in SAO (Z) stored at 0°C for 3 days showed that the Na^+,K^+-ATPase of SAO (Z) red cells was about 3 times as active as that of the normal control (Table 3). This elevated activity is similar to that observed with the red cells of SAO (SG) stored for 2 days at 0°C, suggesting that the increased Na^+,K^+-ATPase activity in freshly drawn SAO (NG) and SAO (SG) red cells is not an artefact resulting from the presence of reticulocytes in the samples.

The bumetanide sensitive Na^+,K^+,Cl^- co-transporter activity was found to be within the normal range (0–0.8 mmol (l cells)$^{-1}$ h^{-1}) for all samples in each experiment (Table 3).

3.5. Adenine nucleotide concentrations in SAO red cells

The concentrations of ATP, ADP and AMP were measured in freshly drawn SAO (NG and SG) and control whole blood. The concentrations of the individual adenine nucleotides in fresh SAO (NG and

Table 4
Adenine nucleotide concentrations in SAO and normal blood

Fresh whole blood concentration [mmol (l cells)$^{-1}$]			
Sample	[ATP]	[ADP]	[AMP]
SAO (NG)	1.02 (1.03)	0.25	0.04
SAO (SG)	1.02 (1.03)	0.26	0.06
Control	1.14 (1.18)	0.26	0.04
SAO (Z)	0.81		
Control	0.72		
Normal range [34]	0.63–1.16	0.12–0.25	0.03–0.05

The two sets of SAO samples should only be compared with the adjacent control samples. These were examined in the same laboratory on the same day. Numbers in parentheses are duplicate results in the same experiment.

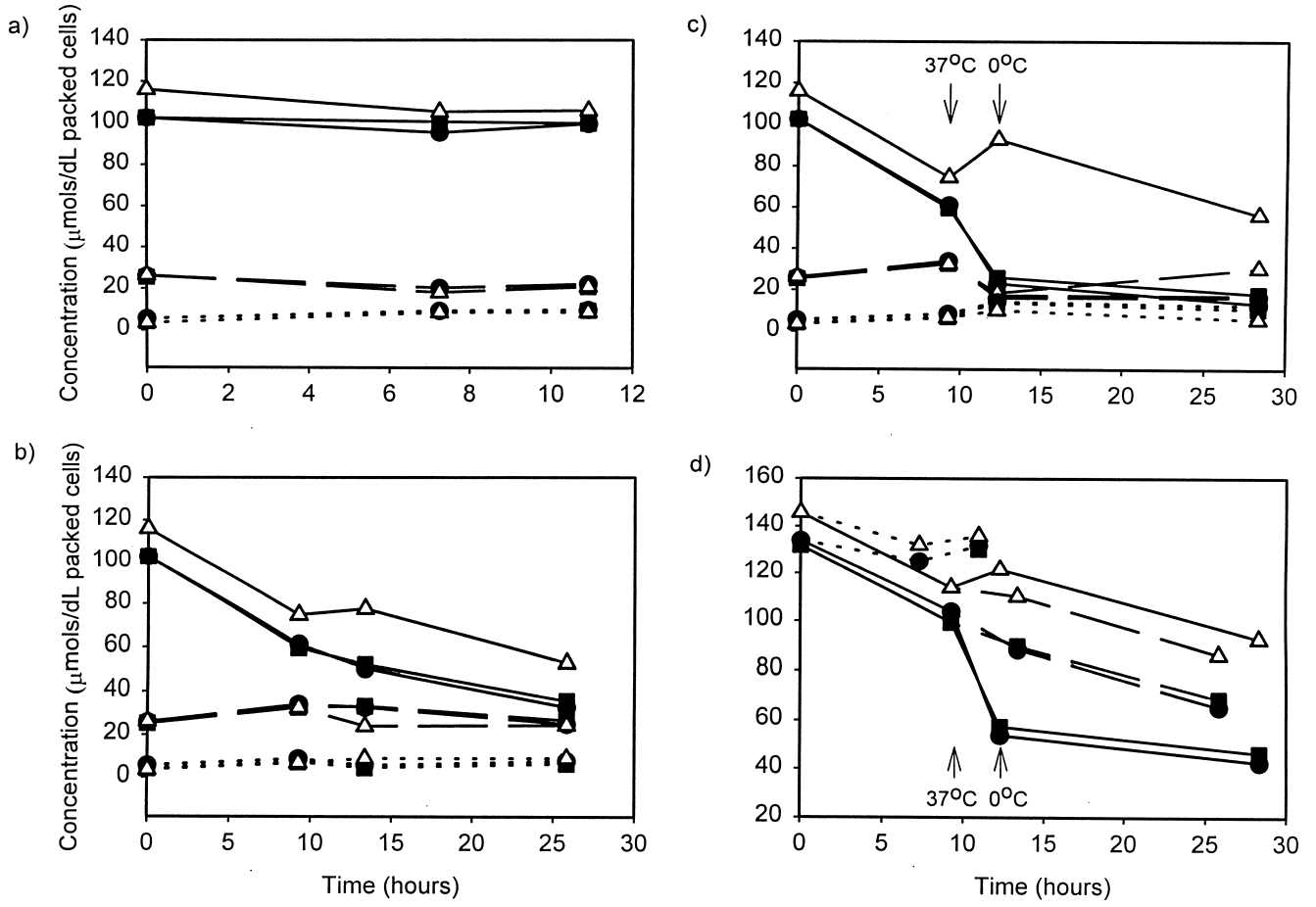

Fig. 4. Concentrations of adenine nucleotides in fresh SAO (NG and SG) and control red cell samples, incubated as whole blood and sampled over 24 h (as described in Section 2). SAO (NG) samples (■); SAO (SG) samples (●); control samples (Δ). (a–c) Continuous lines: ATP concentrations. Broken lines: ADP concentrations. Dashed lines: AMP concentrations. (a) Blood incubated at 37°C. (b) Blood incubated at 0°C. (c) Blood incubated for 8.5 h at 0°C, followed by 3.5 h at 37°C and then 16 h at 0°C. Alterations in temperature are marked with an arrow. (d) Continuous lines: Total adenine nucleotides in blood incubated for 8.5 h at 0°C, followed by 3.5 h at 37°C and then 16 h at 0°C. Broken lines: Total adenine nucleotides in blood incubated at 0°C. Dashed lines: Total adenine nucleotides in blood incubated at 37°C.

SG) whole blood was similar to that found in fresh control blood (Table 4). The concentration of ATP was also measured in freshly drawn SAO (Z) whole blood and was similar to that found in fresh control blood measured under the same conditions (Table 4).

3.6. *Changes in intracellular cation and ATP concentrations on warming precooled SAO red cells*

Many red cell assays, e.g. malarial parasite invasion, ion transport measurements, are performed at 37°C. It is also common practise to incubate cold-stored red cells with glucose at 37°C prior to assay, in order to re-establish the normal ion levels and ATP content of the cells. In order to examine the consequences of such temperature changes we measured the cation and adenine nucleotide concentrations in freshly drawn SAO (NG and SG) and control whole blood after incubation at 0°C for 8.5 h, followed by 37°C for 3.5 h and then 0°C for a further 16 h. Figs. 3 and 4c,d show the results of this experiment.

Incubation at 0°C for 8.5 h resulted in the marked loss of potassium ions and gain in sodium ions in SAO (NG and SG) red cells compared with control

cells (Fig. 3). During this same period there was a fall in ATP concentration, and a slight rise in ADP concentration, in both SAO and control cells, which was more marked in the SAO red cells than the control cells (Fig. 4c).

Incubating the cooled SAO and control whole blood samples at 37°C for 3.5 h resulted in an increase in intracellular potassium and a decrease in intracellular sodium in both SAO (NG and SG) and control cells. However, the SAO (NG and SG) red cells did not regain the sodium and potassium concentrations of SAO red cells in freshly drawn blood (Fig. 3). During this same period of incubation (37°C for 3.5 h) the control blood sample regained almost half of the ATP that was lost during the initial 0°C incubation (Fig. 4c). However, the ATP concentration of the SAO blood samples continued to fall sharply; the ADP concentration of the SAO blood samples also fell and the AMP concentration rose slightly (Fig. 4c).

Returning the SAO and control blood samples to 0°C for a further 16 h resulted in the continued loss of potassium ions and gain in sodium ions in SAO (NG and SG) red cells compared with control cells (Fig. 3). During this same period at 0°C the control blood sample underwent further losses of ATP but only to about half the original control ATP concentration (Fig. 4c). ADP concentration in the control blood sample dropped during the 3.5 h at 37°C then rose again during the 16 h at 0°C (Fig. 4c). However, incubation at 0°C of the SAO blood samples resulted in the further reduction of ATP concentration, with final values being SAO (NG) 0.18 mmol (l packed cells)$^{-1}$; SAO (SG) 0.13 mmol (l packed cells)$^{-1}$.

Examination of the total adenine nucleotide concentration throughout this experiment shows that the control whole blood sample was depleted of total adenine nucleotides during incubation at 0°C for 8.5 h, partially regained the lost adenine nucleotides during incubation at 37°C for 3.5 h, and suffered further depletion on incubation at 0°C for 16 h (Fig. 4d). About one third of the total adenine nucleotide concentration found in the freshly drawn control blood was lost during the three incubations. In comparison, the SAO whole blood sample was depleted of total adenine nucleotides during each of the incubations, with incubation of the cooled SAO whole blood sample at 37°C resulting in the sharpest loss of total adenine nucleotides (Fig. 4d). More than two thirds of the total adenine nucleotide concentration present in the freshly drawn SAO blood was lost during the three incubations.

Incubation of SAO (NG and SG) and control blood at 37°C for 11 h had little effect on the concentrations of ATP, ADP and AMP found in either cell type (Fig. 4a,d). Incubation of SAO (NG and SG) and control blood at 0°C for 26 h caused a drop in ATP and total adenine nucleotide concentration in both cell types, which was more marked in the SAO cells than the control (Fig. 4b,d). Overall, these results suggest that the levels of adenine nucleotides are normal in fresh SAO red cells, but that progressive loss of adenine nucleotides (ATP in particular) occurs on storage of SAO cells at 0°C which, unlike control red cells, is not regenerated when these cells are incubated at 37°C (under the conditions of this experiment).

This set of experiments, measuring the adenine nucleotide levels after incubation at various temperatures, was done in parallel using freshly drawn, washed SAO and control red cells. Similar results were obtained using fresh whole blood samples and freshly drawn, washed red cells (data not shown), demonstrating that the adenine nucleotide levels measured represent the levels within the red cells.

4. Discussion

The present study was initiated because of the previous observations of Dluzewski et al. [18] which demonstrated that the red cells of SAO (NG) were invaded by *P. falciparum* to a significant extent when studied shortly after the blood was drawn. This was in contrast with earlier studies showing that SAO red cells were almost completely refractory to invasion in vitro [13,14]. Dluzewski et al. [18] also found that the extent of invasion fell much more rapidly on storage of SAO cells than normal cells, and correlated this with a faster decrease in intracellular ATP in stored SAO red cells than stored normal cells. Since a substantial proportion of metabolic energy production in red cells is used in maintaining the cellular cation gradients, we were prompted to compare the cation permeability of stored SAO red cells with normal red cells.

In these experiments we examined the properties of SAO red cells from two different ethnic origins (the identical twins, NG and SG, from Mauritius, and the Filipino individual, Z) to determine whether the abnormal membrane permeability properties we observed are associated with the SAO condition. Although NG and SG have jaundice and compensated haemolytic anaemia [19], Z is a healthy individual with a normal peripheral blood reticulocyte count. In all other respects the red cell membrane properties we have studied are indistinguishable in these SAO individuals.

Our results showed that the red cells of SAO individuals had an almost normal intracellular cation content in vivo, but with a small reduction in the level of potassium ions. Storage of SAO red cells at 25 or 37°C resulted in a slightly greater loss of intracellular potassium ions, and gain in intracellular sodium ions, than in normal controls, in the presence or absence of ouabain and bumetanide. In contrast, storage of SAO red cells at 0°C resulted in a much faster loss of intracellular potassium ions and gain in intracellular sodium ions than normal, which was not affected by the presence of ouabain or bumetanide. After only 24 h storage at 0°C the intracellular concentration of these cations approached equilibrium with the cations in the medium. Cold-stored SAO red cells are not only more leaky to both sodium and potassium, but are also more leaky to divalent cations (Ca^{2+}), taurine, and the normally impermeant molecules mannitol and sucrose, than control cells. Although the SAO red cells showed a dramatically increased leakiness to monovalent cations in the cold, SAO cells, which have never been chilled, were also slightly more leaky to monovalent cations than normal red cells. Freshly drawn SAO red cells had a substantially elevated Na^+,K^+-ATPase activity, which is likely to lead to more rapid glucose utilisation and ATP depletion in SAO red cells even when stored warm.

Freshly drawn SAO red cells were shown to have normal adenine nucleotide levels, however on storage at 0°C, the intracellular ATP levels of the SAO red cells decreased more rapidly than normal cells. This confirmed the observations of Dluzewski et al. [18] on the ATP levels in cold-stored SAO (NG) red cells. The loss of ATP which occurred on storage of these cells at 0°C, could not be reversed by incubation at 37°C for 3.5 h in the presence of 10 mM glucose. In fact, incubation at 37°C resulted in further reduction of ATP and total adenine nucleotide levels (Fig. 4c,d). The loss of total adenine nucleotides and the inability of the SAO red cells to resynthesise ATP, when incubated at 37°C in the presence of glucose, may have resulted from the loss of adenine nucleotide metabolites such as inosine, possibly through the leakage of these metabolites out of the cells.

The changes in the cation content of the SAO red cells after incubation at 0°C were not reversed by warming under the conditions of the experiment. This probably reflects the fact that incubation at 37°C caused the already depleted levels of ATP in the cells to fall rapidly, as the Na^+,K^+-ATPase was activated. A further possible reason for this inability to reverse the changes in cation content in cold-stored SAO blood may be that once the cold-induced cation leak occurs on storage at 0°C, the SAO red cell membrane does not revert to the relatively cation-tight state it had in freshly drawn warm blood. The reversibility of the cold-induced cation leak, and the temperature dependence of the leakiness to other compounds observed in SAO cells, need further investigation but we have been hampered by the difficulty in regularly obtaining fresh samples of SAO blood which have been stored warm under defined conditions.

Honig et al. [23] described a Filipino family with stomatocytic elliptocytosis, but no evidence of haemolytic anaemia. Red cells from affected members of this family were elliptocytic with a pronounced stoma, resistant to osmotic swelling in hypotonic solutions and had decreased osmotic fragility. Studies on the cation permeability of these cells at 37°C (which, the report suggests, were maintained at room temperature after bleeding) showed they had a 2–3-fold increase in permeability to sodium and potassium ions in the presence of ouabain [23]. This is similar to the increased permeability to these ions we observed with SAO red cells at 25 and 37°C (Fig. 2d–f). The cells consumed glucose at a rate 60% higher than normal cells. Interestingly, Honig et al. [23] observed a small decrease in the intracellular potassium concentration of the freshly drawn red cells, similar to that found in SAO red cells (Table 1). The morphological and permeability characteristics of the red cells in the

family described by Honig et al. [23] suggest they are also of the SAO phenotype.

There have been several reports of alterations in the properties of band 3 or other red cell membrane properties in SAO red cells. Many of these studies used SAO red cells stored at 4°C, although in some reports the conditions used for storing the SAO cells are not stated. SAO red cells have been shown to be much less deformable than normal red cells [15,16]. The band 3 in SAO red cells has been reported to form a higher proportion of tetramers than normal band 3, [7,24] and shows an increased retention with the red cell skeleton [6,7,24]. Ghosts from SAO red cells show reduced band 3 lateral and rotational mobilities [2,3,24–27] and the intramembranous particles on the P-faces of freeze-fractured cold-stored SAO red cells form unusual linear rows comprising 10–15 particles, [24,25] and these particles are thought to contain band 3. Several blood group antigens show a selective depression in their expression on SAO red blood cells [28], including several antigens and epitopes associated with band 3 [29]. These observations suggest that SAO band 3 has an increased tendency to associate into higher oligomers. The cold-induced leak in SAO red cells may result from the changed associations of SAO band 3 within these aggregates. However it is not clear whether this leakiness occurs directly from the altered structure of SAO band 3, or from the formation of heterodimers between SAO band 3 and normal band 3, or from the interaction of SAO band 3 with some other membrane component.

The cold-induced leak in SAO red cell membranes behaves as a relatively non-specific channel that allows the passage of molecules as large as sucrose across the membrane. It has recently been shown that trout band 3 is capable of forming chloride channels and enabling taurine transport [30]. Malaria-infected human erythrocytes show an increased leakiness to both sodium [31] and potassium [32,33]. It is interesting to speculate whether the latter cation leaks result from a change in the structure of normal red cell band 3 induced by the malarial parasite, which could be similar to a cold-induced change in the structure of SAO band 3 that leads to the increased cation leaks in SAO red cells.

Our results showed that SAO red cells stored for just 24 h at 0°C had properties which were markedly different from their state in vivo. Our experiments were carried out on blood stored in the presence of EGTA. It is likely that during cold storage of SAO red cells in the absence of this efficient Ca^{2+} chelator, the reduced activity of the plasma membrane Ca^{2+} pump caused by intracellular ATP depletion, coupled with the increased membrane permeability to Ca^{2+}, would have rapidly allowed Ca^{2+} entry into the cells and thus activate Ca^{2+}-dependent processes. These would include activation of the Ca^{2+}-dependent K^+ channel (the Gardos channel), which would further facilitate the loss of potassium ions from the cells, and the activation of calpain-mediated proteolysis.

Since it is usual to store and transport red cells in the cold, much of the experimental data reported on SAO red cells kept under these conditions needs re-evaluation using never-cooled or fresh SAO blood, otherwise the data may not reflect the properties of the red cells in vivo. Dluzewski et al. [18] have already demonstrated that the widely quoted observation that SAO red cells are almost completely refractory to invasion by *P. falciparum* and *P. knowlesi* merozoites in vitro reflects the fact that stored SAO red cells have very low ATP levels. These authors showed that SAO red cells are substantially invaded by the parasites when tested a short time after the blood was drawn, so that SAO red cells probably have a normal susceptibility to invasion under in vivo conditions. Our work shows that in addition to the decrease in ATP concentration in the cold-stored SAO red cells, there is gross cation imbalance which probably contributes further to the lack of invasion of cold-stored SAO red cells by the malarial parasites.

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