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Partial Requirements for *in vitro* Survival of Human Red Blood Cells

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Summary. Some of the requirements for survival of human red blood cells were studied *in vitro* at 25 and 37 °C for 1–2 weeks. During the first week at 25 °C in Krebs-Ringer bicarbonate medium with glucose, the cells at 2–5% hematocrit (HCT) maintained normal K^+, Na^+, and water contents with negligible hemolysis. After six days ion gradients decreased, preceded by decline of ATP. With adenosine, ATP was maintained for 1–2 weeks.

Sustained *in vitro* survival of human red blood cells at 25 or 37 °C requires constant pH_o and sufficient substrates to support a glycolytic carbon flux as well as a nitrogen flux via nucleotide turnover. In Earle's salts buffered with HEPES and supplemented with glucose, Eagle's essential vitamins, albumin, and antibiotics, suspensions at 0.1% HCT exhibited constant pH at 7.39 ± 0.03 for at least two weeks at 37 °C. With glucose alone, ATP declined steadily to negligible levels despite constant pH_o, but 0.1 mM adenine supported ATP for one week. Also, several amino acids partially prevented the decline of reduced glutathione during the first week at 37 °C. These results and current knowledge of red cell metabolism suggest a new defined medium for experiments requiring long term incubations, and extend the characterization of human red cell *in vitro* survival to a time period not previously studied.

Key Words red blood cells · sodium · potassium · ATP · glutathione

Introduction

Human red blood cells have long been known to lose cation gradients and to autohemolyze when incubated in serum or artificial media at 37 °C for more than two to three days (Ponder, 1951). K^+ loss, Na^+ gain, and ATP depletion occurring during incubation of whole human blood at 37 °C for up to 90 hr with or without purine additives have been well documented (Bishop, 1961*a*, 1962*a*, *b*; Langley & Axell, 1968; Lionetti, McKay & Gendron, 1968; Chapman, Allison & Grimes, 1971). ATP decline also results in net uptake of Ca^{++}, an ion which induces a rapid selective loss of K^+ (Gardos, 1959) and which hyperpolarizes the membrane (Freedman & Novak, 1983). This change in membrane potential facilitates further passive uptake of Ca^{++}, leading to inhibition of the $(Na^+ + K^+)$-ATPase (Hoffman, 1962), and also to decreased membrane deformability and to extensive echinocytosis (Weed, LaCelle, & Merrill, 1969). Metabolic depletion also is associated with formation of lysolecithin, thus resulting in increased osmotic fragility and autohemolysis (Murphy, 1962; Shohet & Haley, 1973).

A system for incubating human red blood cells for an extended time period at 37 °C would be desirable in order to define the minimum essential requirements for maintenance of normal red cell metabolism and membrane structure and function. In addition, environmental effects on some aspects of cellular aging might be studied, and the metabolic, transport, and volume regulatory characteristics of normal and pathological red cells could be compared. Some success in studying cation and amino acid transport during long term incubations of sheep red cells in serum-supplemented media was reported by Kepner and Tosteson (1972) and by Benderoff, Blostein and Johnstone (1978). This paper describes experiments performed some years ago (Freedman, 1969, 1973; Freedman & Norton, 1975) which attempted to prevent and to understand some of the alterations occurring in human red blood cells during incubation in chemically defined media at 25 and at 37 °C for 1–2 weeks.

Materials and Methods

Cell Incubations

As described below, two techniques were employed, one for initial studies at 25 °C, and an improved method for the more difficult experiments at 37 °C. Sterile technique, solutions, and equipment were used in all experiments.

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Incubations at 25 °C. Fresh human blood from healthy donors was drawn by venepuncture into heparinized Vacutainers and immediately centrifuged at $2,000 \times g$ for 5 min at 4 °C. After aspiration of the plasma and buffy coat, the red cells were poured into Krebs-Ringer bicarbonate medium containing the following: NaCl (120 mM), KCl (4.5 mM), $NaHCO_3$ (15 mM), NaH_2PO_4 (1.5 mM), Na_2HPO_4 (0.7 mM), $MgCl_2$ (0.5 mM), $CaCl_2$ (1.4 mM), glucose (10 mM), Sodium Penicillin G (Squibb, 5,000,000 unit vial, 0.1 mg/ml), Streptomycin sulfate U.S.P. (Squibb, 0.1 mg/ml), and Nystatin fungicide (22 units/ml). For some experiments, the medium was supplemented with 2 mM adenosine, 1% vol/vol fetal calf serum, and 10% vol/vol GIB tissue culture medium (Grand Island Biologicals, *see* Ling & Bohr, 1969, for composition). The medium was bubbled with 95% O_2 – 5% CO_2 to pH 7.5. Suspensions at 2–5% hematocrit (HCT) were incubated in 250 ml Nalgene centrifuge bottles whose polypropylene tops were fitted with rubber Vacutainer caps to allow sampling at desired times via 5–10 ml syringes with needles. All bottles were sealed with parafilm and placed in a constant temperature room in an upright position on a horizontal shaker with gentle shaking. Settled cells were resuspended periodically by means of gentle swirling.

Incubations at 37 °C. Blood was collected as above, except the Vacutainers contained cold sterile acid-citrate-dextrose (ACD) anticoagulant, formula B, pH 4.5, (1 ml/4 ml blood), in which cellular ATP is stable (Beutler & Duron, 1965). The blood was immediately centrifuged for 10 min at $500 \times g$, the platelet-rich plasma was aspirated, and the cells were resuspended in incubation medium and recentrifuged at $2,000 \times g$ for 10 min, followed by aspiration of the supernatant and buffy coat. The incubation medium (Table 1) was filtered (Millipore sterifil unit with 0.22 μm filter), the cells were added, and the pH was adjusted at 37 °C with 0.3 M NaOH or HCl. Use of HEPES buffer eliminated pH drifts encountered with bicarbonate alone. Polypropylene syringes (10 ml or 50 ml) were filled with cell suspension through the port of the receiving flask of the Millipore apparatus and then were sealed with stainless steel or Teflon syringe caps and placed horizontally in an air incubator. Agitation or rotation of the suspensions at 37 °C adversely affected cell morphology. At desired times, syringes were removed from the incubator, the pH's of the suspensions were measured at 37 °C, cellular morphology was checked by phase contrast microscopy, and biochemical analyses were performed on cell pellets collected in microcentrifuge tubes attached to syringes as described previously (Freedman & Hoffman, 1979).

Table 1. Incubation medium for human red blood cells

Earle's salts[1] (mM)	
NaCl	116.4
KCl	5.4
$CaCl_2 \cdot 2H_2O$	1.8
$MgSO_4 \cdot 7H_2O$	0.8
$NaH_2PO_4 \cdot H_2O$	1.0
Glucose	5.6
$NaHCO_3$	15.0
HEPES buffer[2] (mM)	18.0
Streptomycin[1] (units/ml)	100.0
Penicillin[1] (units/ml)	100.0
Bovine serum albumin[3] (g/liter)	10.0
Eagle's essential vitamins[4] (mg/liter)	
D-Biotin	0.2443
D-Calcium pantothenate	0.4765
Choline chloride	0.1396
Folic acid	0.4414
Nicotinamide	0.1221
Pyridoxal HCl	0.2036
Riboflavin	0.0376
Thiamine HCl	0.3373
Amino acids[2] (mM)	
Cysteine	0.5
Glycine	0.5
Glutamic acid	0.5
Glutamine	0.5

[1] Microbiological associates (10× concentrate without phenol red or $NaHCO_3$); [2] Sigma Chemical Co.; [3] Sigma Fraction V; [4] Microbiological associates (100× concentrate).

Biochemical Analysis

Potassium and sodium were determined flame photometrically (DU flame attachment, Beckman Instruments, Palo Alto, Calif.) after overnight extraction of weighed packed cells in 0.1 N HCl (Freedman, 1973).

Water content was measured by drying the cells to constant weight at 100 °C.

ATP was estimated as nucleotide triphosphate with phosphoglyceric phosphokinase and glyceraldehyde phosphate dehydrogenase (Sigma Kit 366-UV), or by the firefly luciferase technique (Beutler, 1971).

Reduced glutathione (GSH) was assessed by the method of Beutler (1971). Controls showed that cysteine in the medium spontaneously oxidized to cystine within 1 day and did not interfere with analyses of GSH.

Supernatant hemoglobin (Hb) was analyzed by the cyanmethemoglobin method at 540 nm, or at 418 nm for greater sensitivity to low hemolysis in the 0.1% HCT suspensions, and was computed as percentage of complete hemolysis for a given suspension. The percentage of surviving cells is taken as (100 – % hemolysis).

Values are expressed per gram of wet packed cells, or per gram Hb.

Results

Of the many conditions and media tried, the results below describe first the Na^+ and K^+ gradients, ATP contents, and the percent of surviving cells in a simple defined medium at 25 °C. Difficulties at physiological temperature prompted subsequent experiments to focus on ATP and glutathione maintenance at 37 °C with a more complex, yet still defined medium.

Incubations at 25 °C

Since glycolysis in human red cells is inhibited by 50% when the pH declines by only 0.4 (Murphy, 1960), initial experiments were conducted at 25 °C at 2–5% HCT in Krebs-Ringer bicarbonate medium with glucose – conditions intended to minimize acidification by lactic acid. Fresh tissue K^+ (95 ± 4 μmol/g cells, SD, $n = 19$), Na^+ (14 ± 3 μmol/g cells, SD, $n = 19$), and water (66.1 ± 0.7 g/100 g cells, SD, $n = 15$) all agreed with the normal values re-

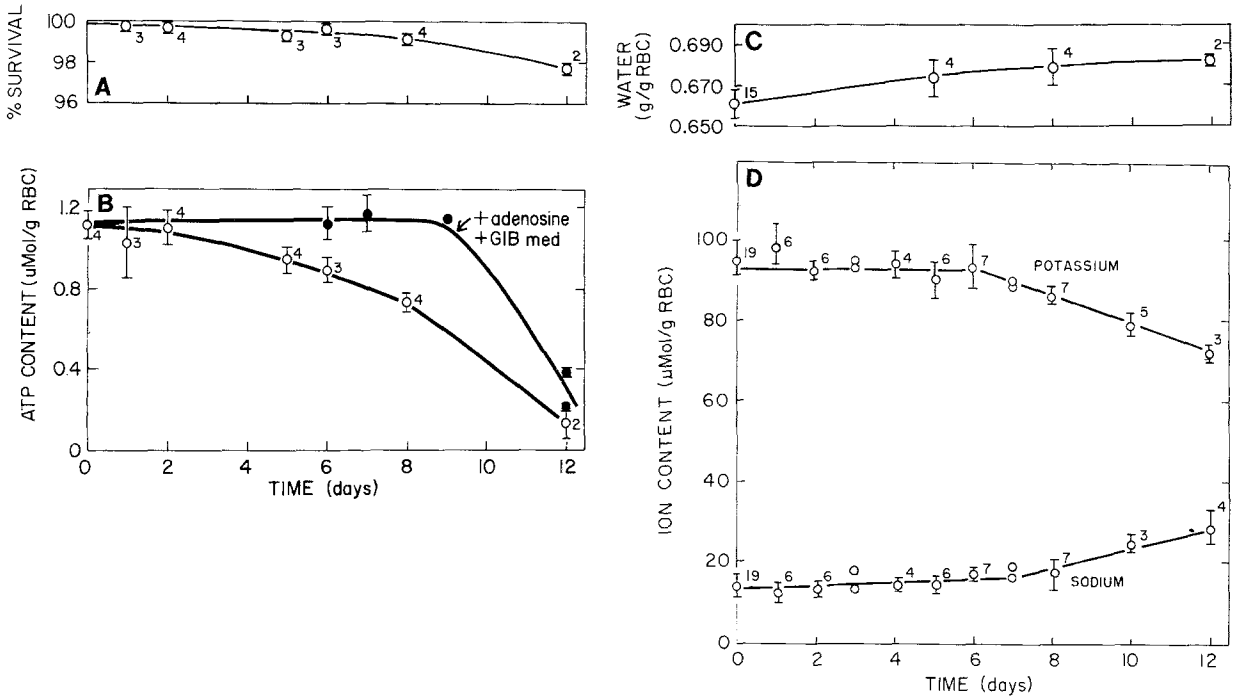

Fig. 1. Percent survival (*A*), ATP content (*B*), water content (*C*), and K^+ and Na^+ content (*D*) of human red blood cells during *in vitro* incubation for 12 days at 25 °C. The cells were at 2–5% hematocrit in Krebs-Ringer bicarbonate medium, supplemented only with glucose (open circles) or also with 2 mM adenosine and with 10% vol/vol GIB culture medium (filled circles, *see* Methods). The straight line segments in *D* were determined by linear regression. Error bars indicate ±1 SD for suspensions from the number of donors indicated by each point

ported by Funder and Wieth (1966). The results in Fig. 1, summarizing data from separate incubations of cells from 7 donors, demonstrate that 99% of the cells survived, that is, did not hemolyze, during the first 8 days *in vitro*. Water contents were within 3% of normal for up to 12 days (Fig. 1 *C*). Normal cell K^+ and Na^+ were maintained for at least 6 days (Fig. 1 *D*), but this time period was insufficient to permit attainment of new steady states or equilibria upon incubation at low temperature, altered external K^+, or in the presence of 10^{-8} to 10^{-4} M ouabain (data not shown).

Despite maintenance of normal K^+ and Na^+ for 1 week, cellular ATP declined from normal (1.1 ± 0.1 μmol/g cells, SD, $n=4$) to negligible levels by the 12th day (Fig. 1 *B*, open circles). ATP was improved to normal levels at between 6 and 9 days by including 2 mM adenosine and also 10% vol/vol GIB culture medium (Fig. 1 *B*, filled circles). In comparable experiments (Freedman, 1973), increasing initial [glucose] from 10 to 20 mM without adenosine did not prevent low ATP on day 12. However, changing the medium (containing adenosine) on day 7, or simply injecting 2 mM additional adenosine on day 9, enabled maintenance of ATP for 12–13 days.

Incubations at 37 °C

Cell survival and the maintenance of normal morphology and ATP contents are technically more difficult to achieve at physiological temperature than at 25 °C. Experiments were conducted with Krebs-Ringer bicarbonate medium, as shown above for the 25 °C incubations, and also with Joklik's medium (*cf.* Kepner & Tosteson, 1972), Hank's salts with HEPES-buffer, and Earles' salts with HEPES buffer supplemented as described in Table 1. In three successive incubations (Fig. 2) using the medium recommended in Table 1, partial substitution of the $NaHCO_3$ with 18 mM HEPES buffer allowed the pH of 0.1% HCT suspensions to be maintained at 7.39 ± 0.03 (SD, $n=33$) for at least two weeks (Fig. 2 *C*). Cell survival averaged 85% at the end of the first week but was only 40% by the end of the second week (Fig. 2 *A*).

At constant pH with glucose substrate alone, ATP steadily declined from normal (4.1 ± 0.4 μmol/g Hb, SD, $n=8$) to negligible levels by the end of the second week (Fig. 2 *B*, filled circles), and the rate of decline was similar to that reported for loss of labeled nucleotides formed after injection of ^{14}C-adenine *in vivo* (Bishop,

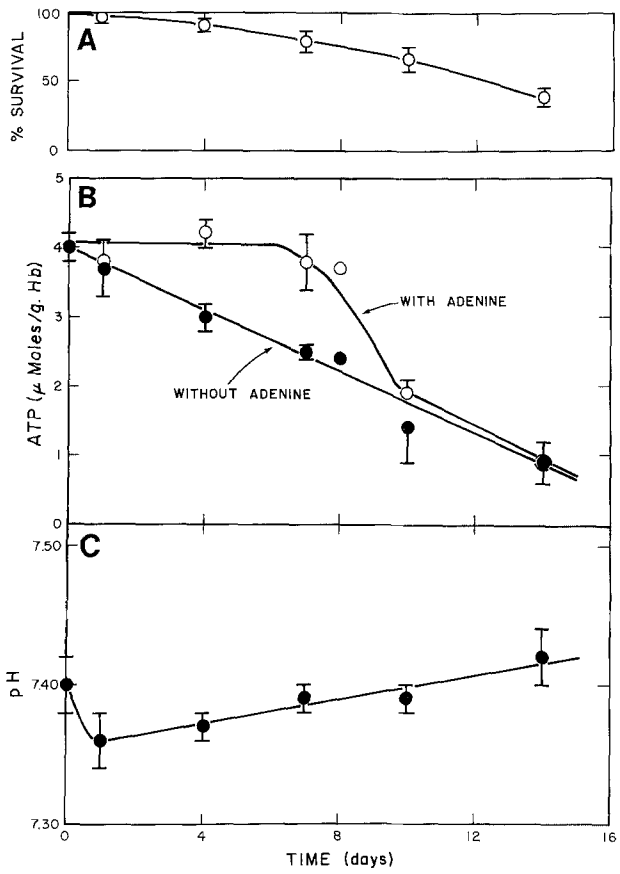

Fig. 2. Percent survival (*A*), ATP content (*B*), and stability of pH (*C*) of human red blood cells during *in vitro* incubation for 2 weeks at 37 °C. The cells were at 0.1% HCT in the medium recommended in Table 1 but without amino acids, and with or without 0.1 mM adenine as indicated. There was no effect of adenine on the pH or % survival. The points represent means and average deviations from three independent paired experiments

1961*b*). With 0.1 mM adenine in the medium, ATP was normal for one week, followed by an abrupt fall (Fig. 2*B*, empty circles). The difference in ATP content with and without adenine was significant on day 4 ($P=0.1$) and on day 7 ($P<0.025$), but the pH and the percent survival were unaffected by adenine in these three paired experiments. In previously reported incubations of whole blood at 37 °C, ATP declined to negligibly low levels in only 3 days (Bishop, 1961*a*). Supplementation of whole blood with purines and purine ribosides provided some initial improvement, but ATP was still only 25% of normal in 3 days (Lionetti et al., 1968). Even under pH-stat conditions (Bishop, 1962*b*), ATP of whole blood declined faster than in the present experiments (Fig. 2*B*).

A final series utilized media with and without added glutamic acid, cysteine, and glycine, each of which is incorporated into GSH – an important reducing agent in red cells (Hochberg & Dimant, 1965). Without amino acid supplementation, GSH was significantly depressed ($P<0.001$) from normal (6.9 ± 0.7 μmol/g Hb, SD, $n=5$) after 4 days (Table 2, experiments B and D). The rate of GSH decline agreed with the half-life expected from the aforementioned amino acid incorporation studies. Including 0.5 to 2.5 mM glycine, cysteine, and glutamic acid in the medium partially prevented GSH decline ($P<0.01$), but did not enable maintenance at its normal level ($P<0.001$). Plasma concentrations of these amino acids (Table 2, experiment D) and additional supplementation with serine alanine, and glutamine (Table 2, experiment B) resulted in similar partial support of GSH. The normal ATP contents of cells in media supplemented both with adenine and amino acids (Table 2, experiment E) indicates that ATP was not limiting for glutathione synthesis. In two experiments, which were at 0.2% HCT in order to collect enough cells for GSH assays, cell survival was 85–90% after 10 days at 37 °C, but the extent of hemolysis was not demonstrably correlated with the presence or absence of amino acids. In these two experiments the rate of hemolysis *in vitro* was comparable to the *in vivo* rate of cell death of about 1% per day.

Discussion

The maintenance of ion gradients, ATP, and GSH, and the lack of hemolysis during incubation in defined media for 1–2 weeks at 25 and 37 °C all indicate that constant pH and a nutrient medium including at least glucose, a purine, and certain amino acids constitute partial minimal requirements for *in vitro* survival of human red blood cells. While cold-stored bank blood has been extensively studied (*see* Bartlett, 1974), the ion transport studies on human red cells in salt solutions (Ponder, 1951), and on sheep red cells in serum-supplemented medium without added purines (Kepner & Tosteson, 1972; Benderoff et al., 1978), are the only previously reported incubations of red cells at elevated temperature that have gone beyond 1–4 days. Incubations at 25 °C (Harris, 1941) and at 37 °C (Selwyn & Dacie, 1954) with glucose for 1–2 days show only small changes in cell K^+ and Na^+. With glucose and purines added to whole blood at 37 °C, ATP declines within 90 hr after lactic acid production acidifies the medium and inhibits glycolysis (Murphy, 1960), followed by K^+ loss and Na^+ gain (Bishop, 1961*a*, 1962*a*, *b*; Lionetti et al., 1968; Chapman et al., 1971). In a 37 °C

Table 2. The effect of added amino acids on reduced glutathione (GSH) in human red blood cells incubated at 37 °C

Time (days)	Exp't	Adenine	Amino acids						ATP (μmol/g Hb)	GSH (μmol/g Hb)
			Glu	Cys	Gly	Ser	Ala	Gln		
0	A–E	–	–	–	–	–	–	–	4.1±0.4 (SD, $n=8$)	6.9±0.7 (SD, $n=5$)
1	C, D	0.1	0.50	0.50	0.50	0	0	0.50	–	6.5, 7.1
4	B, D	0.1	0	0	0	0	0	0	–	1.7, 1.9
	A	0.1	0.50	0.50	0.50	0	0	0	–	5.0
	D	0.1	0.10	0.10	0.25	0	0	0.50	–	3.5
	D	0.1	0.50	0.50	0.50	0	0	0.50	–	3.2
	B	0.1	1.25	1.25	1.25	0	0	1.25	–	3.4
	B	0.1	2.50	2.50	2.50	0	0	2.50	–	3.8
	B	0.1	2.50	2.50	2.50	0.1	0.1	2.50	–	3.4
	E	0	0.50	0.50	0.50	0	0	0.50	3.5	4.1
	E	0.1	0.50	0.50	0.50	0	0	0.50	5.2	–
	E	1.0	0.50	0.50	0.50	0	0	0.50	5.2	5.6
7	D	0.1	0.50	0.50	0.50	0	0	0.50	–	3.0

Human red cells were incubated at 0.2% HCT in 50-ml syringes in the medium described in Table 1, but with the concentrations of added adenine and amino acids as indicated above. The value shown for ATP and reduced glutathione (GSH) are the means of two or three suspensions. Data are presented for five independent experiments, designated A–E.

chemostat at 33% HCT with continuous perfusion maintaining constant pH and [glucose] in defined medium, ATP declined by 20% in 29 hr; however, with added adenine, ATP was over twice normal (Imarisio, Gendelman & Strother, 1969). The present results demonstrate longer ATP maintenance and cell survival than previously reported for whole blood or high HCT suspensions during *in vitro* incubation.

Ideally, a culture medium for red cells should be based on knowledge of their unique enzymology. From catalogues listing about 140 enzymes reported in human red cells (Friedemann & Rapaport, 1974; Pennell, 1974), a metabolic chart was constructed (not shown). The defined medium recommended in Table 1 takes into account not only the requirement of exogenous purines for synthesis of human red cell nucleotides (Gabrio, Hennessey, Thomasson & Finch, 1955; Lowy, Williams & London, 1962), but also incorporation studies showing that several key metabolic intermediates of crucial importance to the mature red cell undergo molecular turnover and have half-lives of less than one week, much shorter than the 120 day cellular lifetime. ATP itself has a half-life of 7 days *in vivo* (Bishop, 1961*b*). The insufficiency of glucose substrate alone for ATP maintenance at 37 °C at constant pH for one week *in vitro* and the ability of adenine to maintain ATP (Fig. 2*B*) are consistent with many biochemical studies and the *in vivo* evidence for a purine requirement.

Glutathione, with a half-life of 4 days, utilizes glutamic acid, glycine, and cysteine for its synthesis (Hochberg & Dimant, 1965) and evidently also for its maintenance *in vitro* (Table 2). Nicotinamide adenine dinucleotide phosphate (NADP) utilizes nicotinic acid and glutamine (Preiss & Handler, 1958; Jaffe & Neumann, 1965), but its half-life is unknown. Flavin adenine dinucleotide (FAD), a riboflavin derivative, is a cofactor for glutathione reductase, and has a half-life of 6 days (Mandula & Beutler, 1970). Other known vitamin cofactors for red cell enzymes are thiamine pyrophosphate for transketolase in the pentose shunt, and pyridoxal phosphate, for transaminases whose functions, if any, in mature red cells are unknown (Beutler, 1970). In lieu of knowledge of the optimal vitamin requirements, Eagle's essential vitamins were utilized at 37 °C. Membrane phospholipid fatty acids turn over with a half-life of 1 day (Mulder & van Deenen, 1965; Shohet, Nathan & Karnovsky, 1968), but the role of serum lipids in the *in vitro* survival of red cells has not been characterized.

The defined medium in Table 1 is convenient to prepare, is based on current knowledge of red cell metabolism and on data from *in vitro* studies, and is an improvement which may facilitate experiments requiring prolonged incubations. The methods and medium described in this paper were recently utilized to study the development of inhibition by Li^+ of Li^+/Na^+ countertransport during

a one-week *in vitro* incubation of human red blood cells (Ehrlich, Diamond & Freedman, 1983; Ehrlich, Diamond, Fry & Meier, 1983). Beyond the methodological usefulness of the results in this paper, the development of morphological heterogeneity during the incubations at 37 °C suggests that additional membrane-associated mechanisms must be better understood before the problem of maintaining human red blood cells for their entire lifetime can be completely solved.

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