A microtechnique for measuring red cell osmotic fragility of infants

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SUMMARY   Chloramphenicol (*D*-threo-2-dichloroacetamido-1-*p*-nitrophenylpropane-1, 3-diol) added to blood samples did not alter the observed shift in the red cell osmotic fragility curves as the samples were aged *in vitro* for 24 hours at 37°C. Nor was the normal rate of loss of 2,3-diphosphoglycerate, ATP, or glutathione from the red cells affected by the presence of chloramphenicol over the same period. Consequently, this bacteriostatic agent can be added to blood samples taken under non-sterile conditions, such as from the heel of an infant, in order to preserve them from the effects of microbial contamination. In this way red cell osmotic fragility results can be obtained on non-sterile samples after their incubation at 37°C for 24 hours. A miniaturisation of the standard osmotic fragility procedure is described, which allows results to be produced from the small, non-sterile samples obtained by heel-prick of infants.

The osmotic fragility test of red blood cells is well established as a screening procedure in the diagnosis of red cell membrane abnormalities (Whitby and Hynes, 1935; Dacie and Vaughan, 1938). Its application in the diagnosis of hereditary spherocytosis and other haemolytic disorders is well documented (Dacie, 1960). It is of particular value in paediatric laboratory medicine where the early diagnosis of hereditary spherocytosis in the offspring of affected patients is important in their clinical management.

The interpretation of results in these cases is greatly helped by repeating the osmotic fragility measurements after incubating a sterile blood sample *in vitro* at 37°C for 24 hours (Selwyn and Dacie, 1954). In some cases red cells from a patient with hereditary spherocytosis will show an abnormal shift in the osmotic fragility curve only after such treatment, this being a reflection of the excessive loss of membrane lipid from affected cells (Reed and Swisher, 1966; Jacob, 1967) and their higher metabolic rate (Jacob, 1965).

A laboratory problem encountered when producing osmotic fragility results on infant patients is that many paediatricians are reluctant to venepuncture these patients and prefer to obtain samples by heel-prick. Such samples are generally of smaller volume than required for standard osmotic fragility measurements and are not sufficiently free of contamination to be incubated at 37°C for 24 hours without extensive denaturation.

The following work was performed to show that the standard procedure for measuring red cell osmotic fragility (Dacie and Lewis, 1975) could be adapted to cater for microsamples, and that the bacteriostatic agent, chloramphenicol (CAP), could be used to maintain blood samples free from the effects of bacterial contamination without detriment to the red cells.

Material and methods

BUFFERED SODIUM CHLORIDE SOLUTIONS
The currently available protocols (eg, Dacie and Lewis, 1975) were modified since dilution of phosphate buffers with distilled water results in lowered solution pH values. The following solutions were prepared in which buffered saline was diluted with dilute phosphate buffer, pH 7·40.

Solution A
Phosphate buffer 11·2 mmoles/l, pH 7·40. Sodium dihydrogen phosphate dihydrate, $NaH_2PO_4.2H_2O$, 3·50 g was dissolved in approximately 1700 ml deionised water, and the pH of the resulting solution was adjusted to 7·40 with 0·1 mole/l sodium hydroxide. The solution was made up to 2 1, final adjustment of pH was made, and the osmolality was measured. An osmolality of approximately 33 mmoles/kg was observed, corresponding to 0·1% w/v sodium chloride.

Solution B

Sodium chloride, 9·0 g, was dissolved in 600 ml of solution A and the pH was adjusted to 7·40. The solution was made up to 1 litre with solution A, and its osmolality was measured. A value of approximately 315 mmoles/kg was observed, corresponding to 0·95% w/v sodium chloride.

Working solutions

Dilutions of solution B with solution A were prepared as required. The osmolality of individual solutions was calculated from:

$$\text{Solution osmolality} =$$
$$(Os_B - Os_A) \frac{V_B}{V_A + V_B} + Os_A \dots \dots (1)$$

where Os_A and Os_B are the solution osmolalities and V_A and V_B are the volumes of solutions A and B mixed. All concentrations were originally expressed in osmolalities and later converted to the more traditional 'equivalent % NaCl' by the conversion 33 mmoles/kg = 0·1% w/v NaCl.

CHLORAMPHENICOL (CAP)

Forty milligrams chloramphenicol (Chloromycetin, Parke Davis and Company, Sydney) was dissolved in 4·0 ml acetone and made up to 10 ml with deionised water.

ANALYSIS OF METABOLITES

Red cell 2,3-diphosphoglycerate (DPG) was measured by the method of Nygaard and Rörth (1969), which was modified and available as a commercial kit (Calbiochem).

Whole blood ATP was also measured using commercial kit reagents (Calbiochem) based on the method of Kornberg (1955).

Red cell glutathione (GSH) was measured by the method of Beutler *et al.* (1963).

Red cell lysates for the assay of metabolites were prepared from whole blood filtered through cellulose according to the procedure of Beutler (1975). The filtered red cells were washed twice with iced saline and lysed in disodium-EDTA, 2·5 mmoles/l, pH 7·00. Samples of lysate were taken for analysis of GSH immediately on preparation; the remaining lysate was stored at −70°C until required for analysis of DPG.

BLOOD SAMPLES

Blood samples were obtained from adults by venepuncture and divided into two parts. One part was defibrinated and handled under sterile conditions; the other was heparinised with 25 IU lithium heparin (preservative-free) per ml blood and treated with CAP solution, 5 μl for every 200 μl blood. Blood samples of 100-200 μl were taken from infants by heel-prick into heparinised capillary tubes and treated with 5 μl CAP on receipt in the laboratory. Haemolysates for metabolite analyses were prepared, and osmotic fragility curves were measured on all samples within one hour of sampling. Samples containing CAP and sterile, defibrinated samples were also placed in an incubator at 37°C for 24 hours after which the measurements were repeated.

OSMOTIC FRAGILITY MEASUREMENTS

A minimum of 100 μl blood is required. A series of buffered salt solutions of osmolalities ranging from 33 to 315 mmoles/kg were prepared by mixing solutions A and B in different proportions. Solution osmolalities were calculated using equation (1). Three millilitres of each solution was pipetted into a disposable cuvette (1 cm light path × 1 cm × 4·5 cm, Ratiolab cat. 1101) followed by 2 μl blood. The mixtures were shaken and allowed to stand for 30 minutes at 20°C, and the remaining intact red cells were then centrifuged out of suspension at 1200 g for two minutes. The absorbance of the supernant fluid in each cuvette was read in a Unicam SP 1750 spectrophotometer at 415 nm without decanting, using solution B as blank.

Results

It was found that the standard method of osmotic fragility measurement (Dacie and Lewis, 1975) and the current method gave identical results for the same sample at one hour and 24 hours after sampling. Patients included normal adults and infants, and adults and infants with hereditary spherocytosis. A minor difference was observed in the osmotic fragility curves of capillary and venous samples taken from the same individual. Heparinised capillary samples maintained with CAP showed a small increase in median corpuscular fragility of 10-15 mmoles/kg (0·03-0·05% NaCl) when compared with the corresponding venous sample after 24 hours. The fragility curves were identical at one hour after sampling (Figure).

The combined effect of heparin with CAP on the concentrations of some major red cell metabolites was also insignificant. Table 1 lists the findings. No major disturbance of the red cell metabolism was obvious from these results. It is possible that heparinised samples maintained with CAP lost marginally less ATP and GSH than defibrinated sterile samples on incubation at 37°C for 24 hours (Table 1). However, these differences were slight and had no effect on the observed mean corpuscular fragility, nor on the overall shape of the fragility curve.

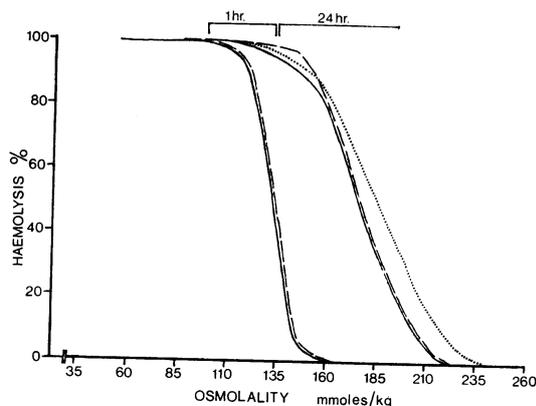Table 2 gives the normal values for the osmotic

Figure   Red cell osmotic fragility curves obtained from a single, normal adult at one hour and 24 hours after sampling: —— venous blood, sterile, defibrinated, analysed according to Dacie and Lewis (1975); — — venous blood, heparinised + CAP, analysed by the described micromethod; · · · · · · · · capillary blood from a finger-prick, heparinised + CAP, analysed by the described micromethod. The curve for the capillary sample at one hour after sampling was superimposable on the venous blood curves and is not shown for clarity.

show an osmotically resistant portion of the red cell population when compared with adult samples, and a wider range of osmotic fragility over the whole population (Danon et al., 1970; Luzzatto et al., 1970). However, it must be noted that in this study adult samples were obtained by venepuncture whereas infant samples were capillary samples.

Discussion

The described work followed from the practical problem of sample treatment for the estimation of post-incubation red cell osmotic fragility in blood samples taken under non-sterile conditions. By far the largest group of samples falling into this category are those taken by heel-prick from infants. These samples are generally of small volume and are certainly not sterile. The standard method of osmotic fragility measurement (Dacie and Lewis, 1975) was modified in two ways to overcome these two problems.

Firstly, a microtechnique was devised whereby only 2 μl blood was required per measurement. The usual heel-prick sample of 100-200 μl was therefore ample to produce two osmotic fragility curves. This diminution of sample volume is possible if measurement of lysis is made at 415 nm. This peak in the visible spectrum of heme is far more intense than the more commonly used peak at 540 nm. Either wavelength can be used without the results being affected.

The second problem, that of preserving non-sterile blood samples against the effects of microbial contamination, was overcome by adding CAP to the samples on their receipt in the laboratory. CAP was chosen as the bacteriostatic agent for testing because its metabolic effects are known to be directed primarily against mitochondrial synthesis functions.

fragility curves of infants and adults derived in this study. During the derivation of these normal values, 22 healthy infants of ages 1, 3, and 5 days were studied. No separation of results was made on the basis of patient's age, nor was any trend in results obvious over this time span. Four patients of ABO groups potentially incompatible with those of their mothers were included in this normal range, and their osmotic fragility curves fell within the range established for the remaining infant patients. Osmotic fragility curves on infants did, however,

Table 1   Effect of heparin and CAP on concentration of red cell metabolites after incubation at 37°C

Patients	Incubation (hours)	GSH (μmoles/l RBC)		DPG (mmoles/l RBC)		ATP (μmoles/l blood)		Mean corpuscular fragility (mmoles/kg)	
		Sterile defibrinated	Heparin and CAP	Sterile defibrinated	Heparin and CAP	Sterile defibrinated	Heparin and CAP	Sterile defibrinated	Heparin and CAP
Normal M	1	2123	1982	4·5	4·8	746	688	138	138
	24	926	1148	1·2	1·1	43	85	180	174
Normal M	1	3022	2946	6·7	6·7	439	569	140	140
	24	535	685	1·7	1·8	7	36	174	168
Hereditary spherocytosis F	1	2483	2379	5·5	5·6	552	569	159	159
	24	576	901	1·0	0·8	60	89	212	218
Pyruvate kinase deficient* M	1	3862	3793	12·4	13·5	405	468	128	128
	24	2388	2889	—	—	72	119	142	148
Pyruvate kinase mutant F	1	3566	3463	6·0	5·8	293	320	128	124
	24	1997	2444	2·0	1·0	17	20	160	160

*14% reticulocytes.
Conversion: SI units to traditional—ATP: 1 μmole/l ≈ 0·059 mg/dl. Salt concentrations: 1 mmole/kg ≈ 0·0303 g NaCl/l.

Table 2 *Normal values for osmotic fragility curves obtained on heel-prick samples from 22 infants (up to 5 days old) and venous samples from 19 adults, before and after incubation of samples at 37°C for 24 hours. All samples were heparinised blood treated with CAP.*

Salt concentration		Infants % lysis		Adults % lysis	
mmoles/kg	Equivalent NaCl (g/l)	1 hour	24 hours	1 hour	24 hours
45	1·4	99-100	99-100	99-100	99-100
60	1·8	97-100	98-100	97-100	98-100
75	2·3	95- 99	97-100	96-100	97-100
90	2·7	92- 98	96-100	95-100	97-100
105	3·2	82- 93	94-100	93-100	96-100
120	3·6	56- 81	87- 99	86- 98	94- 99
135	4·1	23- 54	67- 95	28- 87	85- 98
150	4·5	6- 21	46- 86	9- 43	65- 95
165	5·0	0- 5	27- 68	2- 12	38- 84
180	5·5	0- 1	13- 46	0- 4	12- 62
195	5·9	0	5- 28	0- 2	2- 35
210	6·4	0	2- 17	0- 1	0- 17
225	6·8	0	0- 11	0	0- 7
240	7·3	0	0- 7	0	0- 3
255	7·7	0	0- 4	0	0- 1
270	8·2	0	0- 2	0	0
285	8·6	0	0	0	0

Mitochondrial protein synthesis is inhibited by CAP (Kurtz, 1974). Δ-Aminolevulinic acid synthetase activity is altered in reticulocyte (Rosenberg and Marcus, 1974) and bone marrow mitochondria (Bottomley and Smithee, 1971) leading to impairment of heme synthesis (Beck *et al.*, 1972). Yunis (1973) observed ultrastructural damage to bone marrow mitochondria as a result of CAP therapy in patients. None of these effects, however, should apply to mature red cells since these have no mitochondria. CAP could be expected to produce no serious disruption of their metabolism. The results shown in Table 1 would support this contention.

More direct effects of CAP on red cell membranes, such as those disruptions caused by certain antineoplastic drugs (Ben-Basaat *et al.*, 1972; Schrier *et al.*, 1974), have not been reported. It is known that membrane renewal depends upon active glycolysis within the cells (Ben-Basaat *et al.*, 1972) and that the type of membrane internalisations produced, for example, by primaquine, have a large effect on red cell osmotic fragility (George *et al.*, 1966).

Similar effects of CAP on red cell membranes, however, were not obvious in the current work. Sterile, defibrinated blood samples and heparinised samples treated with CAP were found to have closely similar ATP content and osmotic fragility curves, both before and after incubation at 37°C for 24 hours.

It is concluded, therefore, that CAP can be used routinely to preserve non-sterile blood samples free from the effects of bacterial action without detriment to the red cells, in normal patients and in patients with hereditary spherocytosis, so that post-incubation osmotic fragility measurements can be performed.

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