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Storage of marine fish erythrocytes in liquid nitrogen

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The preservation of erythrocytes from cod (*Gadus morhua*), saithe (*Pollachius virens*) and mackerel (*Scomber scombrus*) at −196° C was studied using dimethyl sulphoxide (DMSO) as a cryoprotectant. Erythrocyte recoveries of greater than 90% were obtained from all species and cod erythrocytes were stored for eighteen months with insignificant lysis. Larger quantities of blood were stored by removal of plasma from citrated blood prior to the addition of DMSO solution, and by storage of pelleted frozen blood in aluminium canisters in liquid nitrogen. Maximum recoveries of washed intact erythrocytes required thawing of pellets in 12·5% DMSO solution and washing with buffer containing decreasing concentrations of DMSO. Washed erythrocytes kept at 4° for at least two days showed little haemolysis, were morphologically similar to fresh erythrocytes and equally susceptible to the δ-haemolysin of *Staphylococcus aureus*.

I. INTRODUCTION

Birkbeck, Chao & Arbuthnott (1974) have shown that erythrocytes of cod (*Gadus morhua*), saithe (*Pollachius virens*), haddock (*Melanogrammus aeglifinus*) and mackerel (*Scomber scombrus*) are more specific, and 4 to 16 times more sensitive, than mammalian erythrocytes for assay of the δ-haemolysin of *Staphylococcus aureus*. However, for most laboratories, regular supplies of fresh blood would be impractical and use of stored blood would be desirable.

Human erythrocytes for blood transfusion are now routinely stored in liquid nitrogen (Krijnen, Knivenhoven & De Wit, 1971). Fish erythrocytes have been stored at −70° C or in liquid nitrogen using glycerol (Cushing, Ridgway & Durall, 1957; Hodgins & Ridgway, 1964), glucose, polyvinyl pyrrolidone or dimethyl sulphoxide (DMSO) (Hodgins & Ridgway, 1964) as cryoprotectants. However, removal of glycerol from thawed suspensions caused extensive haemolysis. Using glucose, up to 89% of salmonid erythrocytes were recovered from liquid nitrogen, but the half-life of washed cells was only 1–2 h. The storage of many human and bovine cell types in dimethyl sulphoxide has been reviewed by Shannon & Macy (1973) and with this substance Hodgins & Ridgway (1964) consistently recovered over 70% of salmon or trout erythrocytes after freezing and thawing. In this paper we report the conditions necessary for storage of marine fish erythrocytes for at least several months in liquid nitrogen.

II. MATERIALS AND METHODS

COLLECTION OF FISH

The fish used in this work were caught by trawling in the Firth of Clyde. The fish were sorted on deck and selected individuals kept alive in open 200 l plastic baths prior to bleeding.

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COLLECTION OF BLOOD

Fish were killed and blood withdrawn from the dorsal aorta using a syringe and needle; with fish less than 500 g in weight, a 2·5 ml syringe and 23 g needle were used, but with larger fish a 10 ml or 20 ml syringe with 21 g or 18 g needle was taken. After withdrawal, blood was immediately mixed with 3·8% sodium citrate to prevent coagulation and blood from several fish was normally bulked and maintained at 0° C in an ice-filled Dewar flask for transport to the laboratory. Serum samples were obtained by allowing blood to clot in universal containers. Serum was withdrawn after transport to the laboratory, centrifuged to remove traces of erythrocytes and stored frozen at −20° C. In some instances blood was withdrawn from anaesthetised fish (Sandoz MS 222; 0·01% w/v in sea water).

STORAGE OF BLOOD IN LIQUID NITROGEN

Equal volumes of citrated fish blood and dimethyl sulphoxide (15 to 30% v/v in citrate dextrose saline (CDS) solution, Hodgins & Ridgway, 1964), cooled to 0°, were mixed and allowed to stand at 0° for 10 min. Blood samples (0·8 ml) containing DMSO were distributed into 2 ml polypropylene screw cap ampoules (Sterilin Ltd, Richmond, Surrey) which were mounted in aluminium canes and the blood frozen rapidly by immersion in liquid nitrogen (British Oxygen Company 10 litre Vivostat liquid nitrogen storage container).

For storage as frozen pellets, the blood/CDS/DMSO mixture was frozen by dropwise addition into liquid nitrogen in a Dewar flask; the liquid nitrogen was removed by decantation and pellets stored in screw cap aluminium canisters (18·5 cm × 3·5 cm diameter, with 1 mm holes drilled at intervals along the length to allow free exchange of liquid nitrogen).

ERYTHROCYTE CONCENTRATION IN BLOOD

The packed cell volume of citrated blood samples was measured using the microhaematocrit technique. A heparinised haematocrit capillary-tube (Sherwood Medical Ind. Inc.) was filled with blood, one end was sealed with ' Cristaseal ' (Gelman-Hawksley), the capillaries centrifuged (2000 rpm, 10 min, MSE bench centrifuge, microhaematocrit head assembly) and the percentage packed cell volume measured from the relative lengths of the erythrocyte and plasma columns (MSE Microhaematocrit reader). Erythrocyte concentrations in citrated blood were adjusted by removal of the calculated volume of citrated plasma after centrifugation (500 g, 5 min).

RECOVERY OF FROZEN BLOOD

Ampoules of frozen blood were removed from their storage canes and rapidly thawed at 35° in a water bath. Pelleted blood was recovered by pouring pellets into a universal bottle which was immersed in a 35° water bath to accelerate thawing.

MEASUREMENT OF THE DEGREE OF LYSIS ON STORAGE

The amount of free haemoglobin in the supernatants of erythrocyte suspensions was used as a measure of lysis. Blood stored in ampoules was thawed at 35° and 0·3 ml added to 20 ml CDS solution containing the same concentration of DMSO as the stored blood. After centrifugation (700 g, 5 min) the supernatant optical density at 541 nm was measured in a Pye Unicam SP600 spectrophotometer. A 100% lysis standard was prepared by addition of 1–2 mg saponin (BDH) to the blood suspension and centrifugation to remove erythrocyte debris.

$$\text{The percentage lysis} = \frac{E_{541}\ \text{sample}}{E_{541}\ \text{after saponin lysis}} \times 100$$

Pelleted blood was weighed into a tared universal bottle and CDS/DMSO solution added to give a 1% w/v suspension; erythrocyte lysis on storage was then determined spectrophotometrically, as described above.

ERYTHROCYTE WASHING

Dimethyl sulphoxide was washed from fish erythrocytes by centrifugation and resuspension in 10 volumes of CDS/DMSO solution containing decreasing proportions of DMSO (12·5%, 10%, 7·5%, 5·0%, 2·5% and 1%) before washing twice in CDS solution.

III. RESULTS AND DISCUSSION

Washed cod erythrocytes suspended in a variety of buffers were stable for approx. 3 days at 4° C. Citrated whole blood was more stable; less than 1% erythrocyte lysis occurred after 7 days' storage at 4° C, but lysis increased during the second week of storage. Citrated haddock, saithe or herring blood were not visibly lysed after one week at 4° C. Blood stored at −20° or −70° using either glycerol (10–20%) or DMSO (7·5–15% v/v) lysed rapidly on thawing. Experiments were therefore done to determine the conditions for storage of fish blood in liquid nitrogen using dimethyl sulphoxide (DMSO) as a cryopreservative.

When cod blood was stored in ampoules in liquid N_2 the optimum concentration of DMSO in the freezing mixture was 12·5%. Storage for 1 month in 12·5% DMSO resulted in 1·5% lysis (mean of 10 determinations). Cod blood stored in 7·5 to 15% DMSO for up to 5 months showed little increase in lysis on thawing during this period (Table I).

TABLE I. Lysis of cod erythrocytes during storage in various concentrations of dimethyl sulphoxide in liquid nitrogen

DMSO concentration (% v/v)	Lysis (%)* after storage for stated period				
	1 wk	2 wk	1 mth	3 mth	5 mth
7·5	6·2	9·1	6·8	5·5	8·2
10·0	0·8	4·9	2·1	4·7	6·8
12·5	0·0	0·2	0·0	3·3	5·5
15·0	0·0	0·0	0·0	7·2	7·0

* Mean of two determinations.

When citrated blood of saithe, haddock, mackerel and herring was similarly tested (Table II), insignificant lysis of saithe, haddock or mackerel erythrocytes occurred with DMSO concentrations of 10 and 12·5% but extensive lysis of herring erythrocytes occurred especially at the higher concentrations of DMSO. The reasons for poor storage of herring blood were not investigated.

TABLE II. Lysis of erythrocytes of various fish species stored one month in various concentrations of dimethyl sulphoxide in liquid nitrogen

DMSO concentration (% v/v)	Fish species			
	Saithe	Haddock	Mackerel	Herring
7·5	4·2	7·6	6·6	11·5
10·0	1·8	5·8	5·3	21·9
12·5	2·3	3·3	5·2	48·7
15·0	9·1	8·5	9·6	68·9

To maximise the quantity of fish blood which could be stored in liquid nitrogen, three approaches were considered. Firstly, instead of mixing equal volumes of 20% DMSO in CDS and citrated blood, undiluted DMSO or 30–50% DMSO in CDS was added to citrated saithe blood to a final concentration of 10% DMSO. Significantly greater lysis of erythrocytes occurred as the DMSO concentration in the added fluid was increased; least lysis occurred when equal volumes of 20% DMSO

TABLE III. Lysis of cod erythrocytes on washing in CDS solutions containing decreasing amounts of DMSO

DMSO concentration in storage buffer (%)	% lysis during washing in CDS containing the following DMSO concentration (%)								Recovery (%)	% lysis after 18 h in CDS solution (+4° C)
	15·0	12·5	10·0	7·5	5·0	2·5	1·0	0·0		
7·5	—	—	—	9·2	2·0	1·0	1·2	1·2	85·4	6·7
	—	—	—	10·7	1·4	1·9	1·9	0·6	83·5	4·5
10·0	—	—	5·1	2·2	0	1·7	0·7	0·5	89·8	4·1
	—	—	7·4	0·8	0	1·6	0·5	1·1	88·6	2·9
12·5	—	4·4	0	0·8	0	0	0·3	0	94·5	2·8
	—	6·8	2·0	0·2	0·5	0	0·2	0	90·3	2·0
15·0	10·5	2·7	0·9	0·2	0	0·9	3·8	2·7	78·3	8·5
	6·8	2·9	0	0	0	1·3	4·7	3·2	81·1	8·4

in CDS and citrated blood were mixed. Secondly, the erythrocyte concentration was increased by removal of citrated plasma from the blood before addition of an equal volume of 20% DMSO in CDS. With cod, saithe and haddock blood less than 6% lysis occurred when the erythrocyte concentration was raised to 30, 34 and 20% respectively. Thirdly, the blood/CDS/DMSO mixture was added dropwise into liquid nitrogen to form pellets which could be stored in canisters rather than ampoules. Cod, mackerel and saithe blood were satisfactorily stored in this way; cod and mackerel blood were less than 3% and 8% lysed after storage for eighteen and six months respectively.

Blood was routinely stored by removal of plasma to increase the erythrocyte concentration to approx. 70% p.c.v., adding an equal volume of CDS/DMSO and dropping into liquid nitrogen to form pellets. In this way citrated blood from one collection (300 ml) was stored and used over a period of three months without deterioration.

Extensive haemolysis usually occurred if the thawed blood was suspended directly in CDS solution instead of in the CDS-DMSO buffer used for storage. Pelleted mackerel erythrocytes stored in 12·5% DMSO were lysed 25–58% when diluted in CDS but only 7–8% lysed on dilution in CDS + 12·5% DMSO.

Dimethyl sulphoxide was washed from erythrocytes with graded dilutions of DMSO in CDS solution to remove both DMSO and serum proteins. Slight haemolysis occurred during the first three or four washing steps (Table III). The washed erythrocytes showed little haemolysis after 18 h at 4° C (Table III), and could be kept at 4° C for at least two days without visible haemolysis. Stored cod erythrocytes were indistinguishable from fresh erythrocytes by phase contrast microscopy or in haemolysin titrations with staphylococcal δ-haemolysin. With rabbit antiserum to fresh cod erythrocytes identical haemagglutination titres were obtained.

The high recovery (>90%) of washed erythrocytes after storage in liquid nitrogen did not necessitate investigation of other parameters known to affect storage of cells in liquid nitrogen. For cultured mammalian cells a slow freezing rate is necessary for optimum viability (Paul, 1976; Shannon & May, 1973) and for macrophages a more complex regimen may be necessary (Foreman *et al.*, 1977). Also, for more fragile erythrocytes a buffer which better reflects the ionic composition of serum may be required.

Currently erythrocytes pooled from several cod are stored for up to 1 year in this laboratory for routine haemolysin titrations. The high specificity and sensitivity of these erythrocytes for δ-haemolysin probably reflects the composition and organisation of the erythrocyte plasma membrane which is under investigation.

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